The Culturing of Algae

A Symposium

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The Culturing of Algae

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Papers presented at the annual meeting
of the Phycological Society of America,
New York City, N.Y., December 26–31,
1949

Edited by

Jules Brunel, G. W. Prescott,
and L. H. Tiffany

Phycological Society of America

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PREFACE

The papers included in this volume were presented at a symposium on algal cultures held during the third annual meeting of the Phycological Society of America, New York City, December 27-28, 1949. Because of the increasing amount and kinds of interest in the culturing of algae, both in this country and abroad, it was hoped that the symposium papers might be collected and printed under one cover. Dr. Edward M. Redding, Director of Research for the Charles F. Kettering Foundation, appreciating the contributions made by the symposium, generously arranged for subsidizing the publication of these papers by that organization. The American Phycological Society and researchers in the various fields of phycology everywhere are grateful for the assistance that has made this volume possible. The Editors wish to thank Dr. S. H. Hutner of the Haskins Laboratories, New York City, for his appropriate Introduction to the Symposium Papers.

NOTE

The culture of algae as a research tool is expanding rapidly as modern day experimenters become interested in such diverse fields as photosynthesis, plant nutrition, food production, pond pollution, and sea weed industry. Algae as a class are considered the first or one of the first plants to appear on the Earth. Hence this unique position in the plant kingdom makes algae, particularly the blue-green, very interesting objects of biological research because of the comparative simplicity of the organism. Marine algae offer an almost inexhaustible source of certain mineral elements, concentrated from the sea water. *Chlorella* and similar green algae have been used in extensive development work in an effort to show their potentialities as a food which can be cultivated commercially. On the other hand substantial amounts are spent annually in research aimed at information which will make it possible to reduce or prevent the nuisance of algal growth in fresh water lakes and ponds.

The Charles F. Kettering Foundation is interested in the use of algae in determining the elements which participate in the photosynthesis reaction. The possibility of culturing many types of algae in test tubes, flasks or drums, greatly simplifies laboratory procedure. Nutrient solutions can be completely inorganic without the necessity of such unknown quantities as soils and soil extracts.

It is felt that this volume of papers on Algal Culture will do much to encourage research in many fields.

C. F. Kettering

May 22, 1950

FOREWORD

Since the days of Pasteur the necessity of pure cultures in the study of bacteria and fungi has been a truism, and the success of pure culture methods is also universally known. Although the same methods have been applied to the growing of microscopic algae for over a half-century by several workers, and although there have been fair to excellent results, it cannot be said up to now that the pure culture technique has played as important a rôle in phycology as it has in bacteriology and mycology.

It seems to me that we are now on the threshold of a new era in the study of chlorophyll-bearing microorganisms, however, and that the years to come will witness a furtherance of our knowledge concerning algae which will parallel the amazing progress made in the study of fungi in the first half of the present century.

This will be due mainly to the more generalized use of pure cultures, to the extension of that method to a number of genera which have never been so cultured before, and to the application of practical and tested methods of large-scale culturing of species of economic importance. Furthermore, as it has in the past, the culturing of both microscopic and macroscopic forms will continue to clarify problems related to taxonomy, morphology, physiology, and life cycles of algae.

There is not much risk in predicting that some microscopic algae eventually will prove as important to mankind as Saccharomyces, Penicillium, Aspergillus, *and* Streptomyces. *If this little book, however incomplete, contributes toward the advent of that day, the authors and editors will feel completely repaid.*

Jules Brunel, *President*
Phycological Society of America

April 21, 1950

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INTRODUCTION

The Rôle of Algal Cultures in Research

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Haskins Laboratories, New York, N. Y.

The cultivation of algae is entering a period of broader relevance to the problems of modern biology. The special value of algae as research materials for fundamental studies may be judged more clearly against a background of developments in adjacent fields.

Nineteenth-century biologists were quickened in their investigations of morphology by the hope, voiced by T. H. Huxley, of discerning the framework underlying the protean variety of life. The longed-for insight into the genealogical aspects of evolution was later extended by geneticists to the mechanisms of evolution. With the rise of biochemistry, the older methods of defining evolutionary changes were broadened by such masters as Kluyver and Warburg to include a search for the key reactions governing the chemical activities of life. The fruitfulness of the unified biochemical-morphological approach is borne out by the work of Kluyver's pupil, van Niel. Van Niel's comprehensive theory of photosynthesis is an essential element in the natural classification of chemosynthetic and photosynthetic bacteria *vis-a-vis* other autotrophic organisms such as green plants. A similar point of view is represented by Lwoff's studies on algal flagellates.

Growth depends on integrated chains of biochemical reactions. Certain of these are now being dissected, link by link. The increasing awareness that many of these links are common to organisms usually considered to be only remotely related to each other, is breaking down the old barriers between zoology, botany, and protistology, created by a predominantly morphological tradition: the test of whether a particular metabolic pathway is to be studied in a protist or in the cells of a cellular plant or animal, is convenience. The careful morphological studies of the past are invaluable in providing a wide choice of well-described species for experimental use. The rapidly-expanding science of fundamental biochemical mechanisms, loosely termed "cell physiology," is little concerned with the devices—the hormones, and nervous, circulatory, and excretory systems—coordinating the activities of the cells of cellular organism; it deals instead with the autonomous activities of cells and non-cellular organisms.

Biologists today are unable to explain satisfactorily the inner mechanisms of such processes, to name but two, as photosynthesis and neoplastic growth. Knowledge of both must be drawn from the same fund of data on cell physiology. This is not to imply that cancer is entirely a cellular process; it is well-known that certain tumors are under a degree of hormonal control, e.g. tumors affecting secondary sex characters. But all neoplasms have cellular proliferation as their basis. To understand abnormal growth one must understand normal growth. Photosynthesis is a vitally interesting reaction not alone for its obvious economic significance as the main generator of energy for life, but also because many of the components of the photosynthetic apparatus are closely akin to or identical with components of heterotrophic systems. The solution of such complex problems as photosynthesis or cancer is not to be achieved by an exclusively frontal attack. As pointed out by Darlington (1948), the electric light was not invented through attempts to improve candles. Every means of extending knowledge of the cellular basis of growth must be called upon. The algae allow an attractive simplicity in the design of experiments on photosynthesis, nutrition, and genetics—all subjects which contribute to the understanding of growth.

It has been remarked by Dr. Bernard Davis that there are no blind alleys in biochemistry. Recondite items of biochemistry have a way of contributing to essential later developments. A classical example is F. Gowland Hopkins' investigations of the pigments of butterfly wings, which materially assisted the elucidation of the structure of folic acid. This emphasizes that one danger in demanding immediate returns from research—i.e., research of the so-called *planned* variety—is that it destroys certain powerful incentives and leads to a creeping paralysis in genuine experimentation. Most pioneering investigations are bedeviled by one obstacle after another. This certainly holds true for the majority of attempts to obtain new types of algae in pure culture. Yet the very existence of these difficulties indicates the possibility that such organisms, once brought into cultivation, can be used for studying metabolites or the adaptations for life under unusual conditions; these opportunities would escape notice or the phenomena in question would be inaccessible to experiment if work were restricted to well-established laboratory organisms. It is the invaluable service of a pioneer investigator such as E. G. Pringsheim that he has, despite formidable technical difficulties, succeeded in culturing many strains of algae belonging to unfamiliar groups and has generously made his cultures available to all interested workers, instead of following the popular course, successful over a short term, of studying familiar variables in familiar organisms.

It may be wondered what it is that engenders perseverance despite these uncertain returns. The career of Beijerinck, as described in the memorial by van Iterson, den Dooren de Jong, and Kluyver (1940), is instructive. Beijerinck, a many-sided genius who was the first to obtain pure cultures of algae, had, as the most marked trait of his scientific personality, a passion for experiment. But also, in the words of his biographers, he was ". . . apparently attracted by those microbes which display beautiful colours in their cultures. . .," and "the blue-green sheen of the cultures of luminous bacteria may be at least partly responsible for the persistency with which Beijerinck studied these organisms." His biographers conjecture, furthermore, that Beijerinck's sense of smell may have led him to study organisms producing attractive or in other ways characteristic odors, as for instance the ester-forming yeasts. The physical beauty of many algae, and the intellectual appeal of the problems which they illuminate, effectively join in counteracting much discouragement in experiment; it may be well to be reminded that scientific investigators are not the ENIACs incarnate of popular legend even in the act of choosing experimental materials.

Algae and Tissue Culture

The foregoing generalizations may be lent some concreteness by considering a sector of the frontier of research which, on the surface, shows very little connection with the growing of algae.

Tissue culture provides an unusally direct approach to the study of cancer. Factors that determine the differentiation and controlled growth of cells and tissues may eventually become isolable as conditions for *in vitro* growth become better defined. The mode of action of carcinogens is representative of the kind of problem that can probably be attacked more successfully this way than by *in vivo* studies. Earle *et al.* (1943) discovered that apparently normal fibroblasts of mice, when grown in a medium of chicken plasms, horse serum, and chick-embryo extract, spontaneously yielded cultures giving rise to sarcomas when injected into mice. It is obviously of the highest importance that the cause of this transformation be identified. This is likely to be an easier task if the cells can be grown on a synthetic medium. The most nearly adequate synthetic medium for tissue cultures appears to be one elaborated by Morgan *et al.* (1950) for chick embryo tissue. That this medium is incomplete is shown by its inability to allow growth of subcultures. One of the compounds that might be helpful in supplementing this medium is vitamin B_{12}, the antipernicious anemia factor. There is a close agreement between the specificity of the B_{12} requirement of the algal flagellate *Euglena gracilis* and that of the chick, rat, and man (Hutner *et al.*, 1949, 1950). A more energetic pursuit of the growth factors for *Euglena* might have made vitamin B_{12} available sooner for use in tissue culture as well as in practical nutritional therapy. The development of the basal medium used for the assay of B_{12} by means of *Euglena* was guided by the early studies of E. F. Hopkins on the inorganic requirements of *Chlorella.* The beginnings of work on the culture of *Euglena* by other workers and the writer was motivated for the most part by a desire to explore the taxonomic and phylogenetic relationships between the chlorophyllous euglenoids and their colorless, and, in some genera, phagotrophic (i.e. particle-ingesting) relatives. These interrelated investigations are not mentioned merely to indicate the demands made by a phycological problem on academic curricula: they indicate the variety of points of view needed in cell physiology and cancer research.

One index of the advance of biochemistry is the rate of identification of new metabolites. Many important compounds first came to notice as vitamins for animals or microorganisms. There are indications that we are coming to the point where all the vitamins for the rat or mouse are known; avian nutrition may not be far behind. In the future, therefore, a much greater reliance will have to be placed on microorganisms (and probably on tissue cultures also) to reveal new metabolites by means of their functions as growth factors. It is quite likely that specialized cells when grown in tissue culture may require growth factors not required by so relatively hardy and undifferentiated a cell type as the fibroblast. Media for tissue culture are already formidably complex, as are also those for many of the microorganisms (e.g. lactic acid bacteria) used for assay purposes. The algae promise to be a recruiting ground for an endless series of guides to yet-to-be-discovered metabolites and to more adequate methods of quantitative estimation of already identified cogs in the cell machine.

Algae as Biochemical Reagents

One method of isolating algae is to use mineral agar to exclude heterotrophic organisms. This procedure also suppresses algae with heterotrophic requirements (such algae may still obtain the bulk of their carbon by the reduction of CO_2). In contrast, algae isolated by the washing procedure (Pringsheim, 1946) have an opportunity to exhibit growth factor requirements. The prevailing impression that most algae do not need growth factors may prove to be erroneous: the condition found in the euglenoids, where growth factor requirements have been demonstrated in all adequately studied strains, may represent a common condition in other groups. In *Euglena gracilis,* the simplicity of the requirements for growth apart from the B_{12} requirement, imparts a special value to this organism as a quantitative reagent for free B_{12}.

Biochemists acutely need microbiological methods (for reasons of specificity and economy) for measuring *all* the forms of a metabolite. With one notable exception, the microorganisms now employed as quantitative reagents measure only the free ("unconjugated") form of a growth factor. The ciliate protozoan *Tetrahymena,* presumably because it has a small-scale equivalent of a digestive tract, can utilize high-molecular forms of folic acid and essential amino acids supplied as intact proteins. The application of this type of organism to the analytical biochemistry of such vital metabolites as B_{12}, biotin, and pantothenate should help elucidate their roles in the economy of the organism. Several major groups of algae contain species which have chloroplasts (and are probably therefore photosynthetic) and are phagotrophic at the same time. Many, perhaps most, Chrysophyceae display this habit, as do many marine dinoflagellates. Such algae might be expected to have rather complex growth requirements. In a sense, this might prove an advantage in making these requirements the basis of assay methods: the longer the roster of growth factors, the greater the variety of assays that can be done with the aid of the same organism and essentially the same basal medium; the most difficult stage in the development of a microbiological assay method is the provision of a basal medium complete in all respects except for the growth factor in question. Workers in this field have to keep abreast of advances in metazoan as well as microbial nutrition; the subject of the heterotrophic nutrition of metaphyta and of cellular algae has as yet contributed little that is immediately relevant to the development of biochemical reagents, or, more narrowly, to the development of better culture methods for non-cellular algae.

The derangements that initiated the carcinogenic process are probably of great subtlety. Ferreting out these abnormal reactions will require analytical tools of corresponding delicacy. The nutritional requirements of algae displaying saprophytic or phagotrophic tendencies may, as mentioned, provide the source of some of the needed living analytic reagents. It would seem good strategy to work out as completely as possible the requirements of related more nearly autotrophic forms, if such are available; this would minimize the risk of attempting to solve complex problems in organic nutrition while under the handicap of using a basal medium inadequate in respect to essential inorganic nutrients and known growth factors. The vast host of algae with impaired autotrophism hold forth to the vitamin enthusiasts the promise of a vast arena for their talents.

The Growth of Algae in Darkness

One simple experimental approach to the nature of photosynthesis for some curious reason has been neglected: it is the growing of algae in darkness. If we knew what was the first product of photosynthesis (an entity which remains as elusive as Lewis Carroll's Snark), and if organisms were permeable to this substance, it should enable us to grow any photosynthetic organism in the absence of light—i.e. it should bypass photosynthesis. This type of problem is not confined to algae: attempts to grow purple bacteria anaerobically in darkness have failed (they can be grown anaerobically in light, and aerobically in darkness). A penetrating "first product of photosynthesis" might allow growth of bacteria now termed obligately chemo-autotrophic, e.g. certain species of *Thiobacillus* which require oxidizable inorganic sulfur compounds as their energy source. It remains to be seen whether such a panacea for heterotrophic growth actually exists. If exist it does, it would have an importance beyond photosynthesis: the analysis of the ultimate energy store for growth thereby might be carried beyond the stage of the high-energy phosphate bond as represented by such familiar compounds as adenosine triphosphate and phosphoglyceric acid.

The study of photosynthesis has technological ramifications. The growing of *Chlorella,* once carried out entirely in the spirit of theoretical inquiry, may on an industrial scale add to the world's food resources. The success of this new enterprise will depend on the choice of a suitable organism and a knowledge of the conditions for dense growth. Production of dense cultures of *Chlorella* or of any other alga comes perilously close to exhausting the supply of pertinent information on the inorganic requirements for growth. The assessment of the food value of an alga depends on feeding tests with animals. Because B_{12} is nearly lacking in higher plants, and is therefore frequently a limiting factor in those rations for animals which are based on plant sources of protein, the choice of a food alga is by no means simple; the biological value of other constituents of the alga must also be taken into account. The compelling needs of modern biological technologies heighten the necessity of lowering the barriers of academic jurisdiction which separate the various branches of biology.

Conclusions

The growing of algae means more than micro-horticulture under academic botanical auspices: it helps fashion instruments for probing into cell physiology—the common point of departure for attacking such diverse problems as the nature of photosynthesis and of neoplastic growth. To accomplish this, the terms *taxonomist* and *experimentalist* must cease to be mutually exclusive; the blending begun in the field of genetics must be extended to cell physiology. Then both will make the best use of the organisms available in nature. The taxonomist through participation in these enterprises stands to gain new criteria of evolutionary relationships and new insights. Such studies provide a firmer basis also for the technological development of algae as food.

SOME PROBLEMS IN THE CULTIVATION OF ALGAE

H. C. BOLD

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I have deliberately employed a rather nebulous title for this paper because it is my purpose to discuss several aspects of the cultivation of algae which are themselves in need of clarification. It is now almost sixty years since Beijerinck (1890), following the pioneer methods of bacteriologists, mixed his *Chlorella*-containing ditch water with melted gelatine, and obtained pure cultures of the organism by plating. Although great advances have been made since in the technique of cultivation, and important results have been obtained from the study of algae in pure culture, the field is still an open one in need of further development in a number of aspects, but especially in respect to application of methods.

In the first place, it seems to the author that from one point of view we are again in the position of Beijerinck who adopted the techniques of bacteriologists available in his time, but we are not taking advantage of those techniques in applying them fruitfully to some of the problems that confront the modern phycologist. I have reference here, for example, to the classification of many unicellular and colonial algae, especially Chlorophytan forms, the taxonomy of which is nothing short of chaotic. Just as the early bacteriologists naïvely collected and described bacterial species from various habitats, before it had become clear that the real criteria of taxonomic delimitation must be obtained from prolonged morphological and physiological studies, so even in this day, many phycologists blithely, and perhaps with equal *naiveté,* collect unicellular algae and name them, often from preserved material in which most of the morphological criteria are no longer recognizable.

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For example, who among us can, with authority or even with reasonable assurance, define the generic and specific limits in such series of organisms as (1) *Chlorococcum, Cystococcus, Hypnomonas, Chlorosphaera* and *Trochiscia;* (2) *Chlamydomonas, Gloeocystis, Gloeococcus, Palmella, Sphaerellopsis* and *Sphaerocystis*? One of my students, Mr. Richard Starr, has now been engaged for a year and a half in studying some seventy-five isolates of "*Chlorococcum*-like" algae. The literature on these forms contains numerous incomplete, fragmentary descriptions which offer few really useful taxonomic criteria. True understanding, classification and identification of such cultures is not achieved by percursory examination, such as might suffice for some of the higher algae. More than thirty years ago Brunnthaler (1915) emphasized this when he wrote of *Chlorococcum,* and I translate: "The genus is entirely unnatural; only a few species are known accurately to any degree. Most species probably do not belong here and instead may be stages of other algae. It is possible only by cultural studies to establish the membership of a plant in this genus." How many "new" or previously "undescribed" species of unicellular and colonial forms have been "described" since these words were written, with the diagnoses based on chance collections or on observations of material studied in a mixture of other algae, without continuous observation of the various morphological manifestations evoked by changing environmental conditions! The author, himself, has not been without blame in this respect. In a relatively recent issue of a familiar journal a "new species" of a Polyblepharid is described by another phycologist. The illustration of the type depicts a somewhat inverted conical cell, neatly and uniformly stippled except for a suggestion of a pyrenoid, and with the proper number of flagella characteristic of the genus, but otherwise devoid of such morphological details as stigma, contractile vacuoles and nucleus, omissions which are not supplemented in the text.

It is often argued in some quarters that preparation of herbarium specimens properly labeled and deposited in suitable herbaria, would form a firmer basis for such work. Whereas none of us would deny the importance of herbarium specimens, especially of the larger algae, it is true that for many of the plants at present under consideration, such specimens fail to preserve the critical taxonomic features such as flagella length and position, number and position of contractile vacuoles, relation of flagellar plane to that of the stigma, etc. Another student of mine, Mr. Walter Herndon, is currently studying a species of *Pleodorina,* with beautifully ribbed chloroplasts, between the arms of which occur alternately a number of contractile vacuoles. Herbarium specimens and preserved material fail to help us decide whether Shaw's (1894) omission of them in his drawings was due to their actual absence or to incomplete observation of his material of *P. californica* Shaw. My point may be emphasized further perhaps by two examples, one involving the author. About twenty years ago as a young student he had occasion, under the gentle guidance of the late M. A. Howe and T. E. Hazen, to examine herbarium specimens labeled *"Chlorococcum."* Even under such expert tutelage the specimens were not particularly convincing to any of us, but with the assurance of youth the author wrote (Bold, 1931): "In cell size these specimens[1] are in agreement with the material I have worked with,[2] and their aggregation of angular cells into a loose coenobium-like mass seems to confirm the identification I have made." Today he blushes at his *naiveté* for, in his subsequent experience, he has observed that almost any unicellular alga, in crowded conditions, will behave in similar fashion. More recently a student engaged in a survey of the algal flora of a particular geographical region wrote the author as follows with reference to gross, non-unialgal cultures of *Chlorococcum*-like algae he had been studying: "The massive chloroplast is granular. 'Granular' may not be the word—, but it is not a smooth green—, uneven, almost cracked, perhaps. The pyrenoid is pretty well concealed, and I believe that I have detected more than one, but under the circumstances would hesitate to swear whether it was a nucleus or pyrenoid." This zealous individual, probably through no fault of his own, is apparently unaware that the appearance of the chromatophore of algae varies markedly under different physiological conditions, a fact clearly emphasized by Czurda (1935) and verifiable by continuous study of algae in cultivation.

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[1] Specimens of *C. infusionum* collected by N. L. Gardner and F. S. Collins.

[2] Bold, 1931.

Perhaps these remarks and examples may serve to emphasize the author's studied opinion that phycologists, insofar as many of their investigations of lower algae are concerned, are at about the stage of the early bacteriologists who confidently described new species of bacteria on the basis of morphological criteria alone. A similar situation exists in the fungi as a result of activities by some of our mycological colleagues especially those in medical mycology. There is, however, an optimistic aspect of this problem: in many cases, bacteria and dermatophytes fail to provide sufficiently distinct morphological criteria for the recognition of the individual forms or species. Even in such difficult algal genera as *Chlamydomonas, Chlorococcum, Chlorella,* and many soil algae, careful, prolonged and repeated observation of the organisms maintained in laboratory cultures usually reveals sufficienly clear morphological criteria to effect species-differentiation. It well may be that further work in such genera, particularly *Chlorella,* will indicate that the solution of the taxonomy of these forms must be based to some degree on physiological criteria obtained from their study in pure culture.

The problem of segregating such genera as *Chlamydomonas, Palmella, Gloeocystis* and *Gloeococcus* and other Tetrasporalian forms has been mentioned previously. Comparative cultural studies under controlled conditions correlated with field observations will, in the author's opinion, more rapidly provide a satisfactory solution than examination of countless herbarium specimens. The crux of the problem here is the degree and duration of motility as well as structure and manifestation of the gelatinous layers in which the cells are encapsulated. The extent to which the external environment is responsible for evoking these diagnostic criteria has never been adequately investigated.

Most of these genera of Chlorophyta yield readily to cultivation. The unicellular Chrysophyta (Chrysophyceae) and Pyrrhophyta will be discussed in a later portion of this paper, but this leads me to an important aspect of my topic—: how can such pure- or at least uni-algal cultures be preserved for future use in taxonomic work? Again we are lagging behind the bacteriologists and even the mycologists who have maintained for many years a collection of type cultures. Dr. Pringsheim's collection formerly at Prague and now at Cambridge, the collection now located at Charles University in Prague and the Bourrelly cultures in Paris represent the European collections. From correspondence with Dr. Pringsheim there seems to be doubt that his collection will long survive him, and present conditions in Czechoslovakia are not encouraging. It seems to me, as has been suggested previously by members of this Society, that it is our responsibility somehow to establish a permanent collection of pure cultures of living algae in this country. With the current great increase in use of algae for genetical and physiological studies both by private investigators and government research establishments, some financial support and encouragement for such a project must be possible.

The remaining "problems" involved in or related to the cultivation of algae, to which I shall now turn my attention, are more in the nature of "sins of omission" rather than some of the taxonomic "sins of commission" we have just reviewed. There probably is not available such an assemblage of plants as favorable as the algae for providing so many investigators with challenging opportunities for research. The writer has several times interrogated plant physiologists as to whether normal, metabolically active algal cells secrete into the surrounding medium some of the sugar presumably formed during their photosynthesis. Their responses have been vague and indicate lack of pertinent data. This is a question of fundamental importance for, inasmuch as we know from the work of Moewus and Kuhn that more complex compounds are actively secreted by healthy algae, on theoretical grounds there is no reason why one should not expect some diffusion of photosynthate into the external medium. If this does occur it is a factor which has been largely overlooked in limnological and ecological studies of microorganisms; if it does not occur, one is confronted with another apparent exception to the phenomenon of diffusion as it relates to living cells. Such basic problems as these are readily approachable by the technique of pure algal cultures.

The persistent and insistent report of nitrogen fixation by certain Cyanophyta indicates the need for further study of this group and their activities particularly in soils, while the occasional faintly whispered claims of a similar nature for certain soil-inhabiting Chlorophyta merit studies of their rôle in the soil flora. Furthermore, the Cyanophyta afford an excellent field for the application of pure culture techniques in both morphological and physiological studies. Only a start has been made, for example in such recent studies as those of Fogg (1944, 1948), in obtaining data to explain the significance of heterocyst and akinete formation. Very little is known of the germination and subsequent development of these reproductive cells—data which again could be obtained through the application of pure culture techniques. Finally, the important studies of van Niel (1935) on the photosynthesis of pigmented bacteria suggest that basic data may be forthcoming from a study of photosynthesis of Cyanophyta grown in pure culture.

In the field of algal physiology little is known about the nutrition, growth requirements and metabolism of such groups as the Chrysophyta, Pyrrhophyta, and Euglenophyta. With the exception of a few genera of the last two groups scarcely any have ever been studied in uni-algal or pure cultures.

Although we hear and read a great deal about studies of sexuality and its genetics and physiology in *Chlamydomonas,* how much more might be learned from similar studies of diatoms, desmids and filamentous genera! Cytological observations would undoubtedly increase our knowledge of all these groups, were a continuous and abundant supply of material available in controlled laboratory culture.

Finally, with the modern emphasis on possible augmentation of our agricultural commodities directly or indirectly by mass cultivation of algae, studies of the techniques of such cultivation based on exact knowledge of their nutritional requirements like that obtained by Chu (1942, 1943) indicate a fertile field of endeavor.

It seems to the writer, that whereas the progress in cultivation of algae during the sixty years since Beijerinck's (1890) paper has been edifying, algal cultivation is nonetheless a tool for most phycologists rather than an end in itself. As a group we have been remiss in using the tool where it well might have helped us to finish our own particular job.

THE SOIL-WATER CULTURE TECHNIQUE FOR GROWING ALGAE

E. G. PRINGSHEIM

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INTRODUCTION

The soil-water technique for growing algae can be traced back to Goeppert and Cohn's culture of *Chara* in aquaria with soil in the bottom, more than a century ago (Cohn, 1901). These famous botanists, observing among their *Chara* cuttings many other algae which they had not introduced, investigated them and so began (1849) the CRYPTOGAMENFLORA VON SCHLESIEN, one of the first studies of its kind on the Continent. The innovation of my two fellow citizens was not followed immediately, but was never quite forgotten. I remember my professor, George Karsten, cultivating diatoms in beakers with soil on which boiling water had been poured.

Another line of development began with Beijerinck's (1901) observations of the algal flora growing in water to which different amounts of soil had been added, leading to a preponderance of one algal group or another, according to the quantity of the solid substrate. By adding fibrin, Jacobsen (1910) obtained enrichment cultures of polytrophic flagellates, a method subsequently adopted by Kniep and Buder and their co-workers in Germany. (vd. Schreiber, 1925; Bolte, 1920).

Repeating Jacobsen's experiments, I tried in 1919 to obtain subcultures of a single species by first pasteurizing soil covered with water and inoculating it. This was done by employing fine pipettes, probably first used by Klebs. Individual cells were drawn in under a microscope and blown out into test tubes containing putrifiable matter, soil and water. By covering bits of cheese or wheat grains with soil it was possible to restrict putrefaction to the bottom of the tube and so provide cleaner cultures than with Jacobsen's technique. It was soon found, moreover, that by omitting organic matter other than that contained in the soil, many more algae could be grown in clonal cultures, provided the isolation of algal species was secured by a repetition of the isolation procedure.

The advantages of this bi-phasic soil-water technique were found to be manifold. Its adaptibility permits one to grow almost any species of small organisms, especially algae. Healthy material for morphological investigations and for further culture experiments can be grown with a longevity that surpasses isolates grown in mono-phasic solutions. A strain once put into culture does not get lost easily. The rich and homogeneous growth can be investigated in all of its developmental stages under conditions resembling those of the natural habitat. The technique is easy to employ inasmuch as it does not require bacteriological sterility. It can be employed, therefore, without elaborate equipment and on a wider scale than with the pure culture technique, and by maintaining many strains in test-tubes it permits the comparison of related species.

This technique was described, mainly as a preparatory method before final purification (Pringsheim, 1946). Its adaptability to ecological studies and its application to various needs have been shown in other papers. Herein I shall give an account of further experience.

MEDIA

The imitation of natural conditions will provide a miniature artificial pond. It is not possible in many cases to give in a liquid medium all nutrients required by an alga because many species cannot adapt themselves to other than very dilute solutions. Also, such indispensable elements as calcium, iron, magnesium, phosphorus tend to precipitate. Nutrient solutions of a suitable pH therefore are soon exhausted. In nature substances from the mud-phase replenish nutrients taken up from the water by organisms, and organic compounds keep indispensable chemical elements, especially iron and manganese, in solution. This takes place also in soil-water cultures where the mud-phase serves as an accumulator and a place of reduction and synthesis so that heavy metals are kept in solution. A supply of organic growth factors seems to play a rôle also.

These conditions and relations have the greatest favorable influence in cultures of species from polytrophic waters, whereas those from oligotrophic lakes and running waters are adapted to lower concentrations and cannot be grown in dense populations in a restricted volume of water. It would be wrong, however, to conclude that the soil-water technique is only useful in dealing with algae from polytrophic habitats. By using soil poor in soluble substances and by reducing its quantity (compared with that of the water) the technique can be adapted to species from oligotrophic habitats.

A suitable kind of soil can be found only by trial. Recently-manured loam will seldom be favorable, whereas soil from a garden bed with seasoned compost or leaf mould, or from a well-worked arable field generally will prove to be satisfactory. Without being able to give an explanation, I mention the experience that clay soils in most instances give excellent results that are not obtainable with other soils, just as ponds on clay seem to be rich in interesting forms of algae. Sand without humus cannot replace soil.

The properties of a given soil can be changed by making additions, calcium carbonate being effective in raising the pH, peat in lowering it. But the latter also increases the humus content. Ammonium-magnesium phosphate is useful for its low solubility so that without causing too high a salt concentration, a constant supply of N, Mg, and P is furnished. With acid soils, ammonium-magnesium phosphate is of course immediately dissolved. Clay soil with NH_4MgPO_4 is exceedingly fertile and causes luxuriant growth, for instance, of most Euglenophyta. A few chlorophyll-bearing algae and most of the colorless form are favored by supplying organic substances, such as barley grains or starch covered with soil and water.

All these ingredients should not be mixed with the soil nor put on top of it, but placed at the bottom of the test tubes or other containers. Like the soil itself they are introduced dry so that walls of glass receptacles remain clean. The destruction of contaminating organisms is performed by heating in a steam chamber for an hour on two consecutive days. When material such as peat or wheat which are not easily soaked are used, previous heating in a water bath or the employment of boiling water prevents particles from floating up and soiling cotton plugs.

ISOLATION

The soil-water technique is meant to be used for uni-algal cultures. If mixed populations from nature are introduced, those species which grow rapidly soon prevail, whereas the more exacting, and often interesting ones, are found at best only at the beginning of the culture period. In order to free the more selective species from competition, a mechanical isolation with capillary pipettes under the microscope has been worked out. A binocular dissecting microscope can be used, but is not always sufficient to distinguish the species owing to its poor optical performance and low power of magnification. An instrument with condenser and ordinary low-magnification objectives equipped with porro prisms to erect the image has been constructed. This is far superior in color differentiation and resolution, so that, for instance, species of *Euglena, Trachelomonas,* and *Chlamydomonas* can be distinguished when swimming in a watch glass. Its lower working distance, however, calls for more practice in handling the tip of the capillary than does the ordinary binocular.

Pipettes are made of soft glass tubing with a 3-4 mm. bore. Hard glass has no advantage and is more difficult to handle. Even organisms from acid habitats are not damaged by alkali from the glass during the short period of manipulation. Lengths of glass tubing of 35-40 cm. are drawn out to provide two pipettes which are plugged with cotton and sterilized singly in larger tubes. Before use, the narrow part is drawn out again over a little flame to about 0.5 mm. bore and at a distance of 6-10 cm. again to a real capillary of about 0.1 mm. which is cut short by a sudden pull with a pointed forceps, thus producing a circular, smooth opening. Recent experience has taught that the narrowest part of the pipette should be short in order to give little resistance to the influx of water by capillary force. Then also the blowing out of the droplet of water with the algal cells is easier than with long narrow pipettes as previously recommended, so that a piece of ordinary rubber tubing, closed at one end with a glass stopper provides sufficient expulsion. This is removed for drawing out of capillaries.

The isolation of algal cells is performed by first holding the tip of the capillary over the fluid so that its position is just perceived, and then quickly lowering its open end so that the cell is drawn in, and then immediately lifting it out again. This manipulation can be repeated several times until up to ten cells have been taken up. They are then blown into a few drops of sterile fluid contained in a watch glass which has been sterilized in a Petri dish. By repeating the process once or twice the cells are isolated from other organisms and can then be transferred singly into soil-water tubes. As a washing fluid dilute soil extract is generally suitable.

Cultivation

Because primary cultures are inoculated with one cell only to secure homogeneity, it takes a long time before multiplication develops visible evidence of growth even under favorable culture conditions. In most instances development should be detectable after a month, but no result can be expected if growth is not seen after two months. Primary cultures as a rule are not suitable for a detailed study of the alga because there are too great differences between the oldest and the youngest cells. Subcultures inseminated with as much material as available are much better when inspected young. Frequent subculturing is beneficial for quickly growing species.

No artificial light is as good as daylight near a north window. This of course is not sufficient for many algae in British climate during a great part of the year. A south window with tissue paper as a protection against direct insolation will often serve well during winter. In respect to artificial light I still find an ordinary 500-Watt bulb with water screen better and more economical than luminescent tubes. The heat radiation of the latter is by no means negligible, and their spectral emanation is less in conformity to the absorption of algal pigments than that of incandescent lamps. Moreover a great number of culture vessels can be arranged at equal distances around a bulb, and lower intensities obtained in greater distances; whereas the distribution of light from a number of tubes involves complications.

General rules about light intensity cannot be given. Most Cyanophyta, Chrysophyta, Chrysomonadaceae, the Euglenophyta and the Cryptophyceae, as well as the majority of other filamentous algae cannot be made to multiply more quickly by employing bright light. Many Volvocales, however, maintain a better growth at a high intensity. Most of our strains, on the contrary, are removed from their places of cultivation before they are at the height of their development and are kept in a dimly lighted, cool place.

We have had no experience with cold water algae. Except in hot summers all our algae grow well at "room temperature" (18-22° C. in England) and there is no indication that lower temperatures are better for their multiplication, although most of them live longer when the temperature is not high.

A great difficulty is evaporation. Agar dries out; liquid cultures often produce a growth near the water margin which is lost when the level drops. Caps of wax paper fastened with rubber bands reduce evaporation and soiling by dust. Carbon dioxide seems to penetrate sufficiently, although in soil-water cultures CO_2 is also provided from the mud-base. Tubes with plastic screw caps are still better, provided they have cardboard washers. Their necks, however, make them less useful for soil-water cultures than for agar and liquid media.

Application

More algal species can be grown in soil-water cultures than in all other media combined, among them some delicate ones, although experience is still limited. The following summarizes some of our experiments:

CHRYSOPHYTA (Chrysophyceae): *Ochromonas, Uroglena, Synura, Coccolithophora,* and among colorless forms, *Monas,* *Anthophysa,* and other iron flagellates were grown. As a rule the amount of dissolved organic matter has to be kept low.

CHRYSOPHYTA (Bacillariophyceae): If the media are well adapted multiplication is good. Small species of *Navicula* and *Nitzschia* often overgrow other algae which have not been sufficiently washed.

CHRYSOPHYTA (Xanthophyceae): Every species so far tested could be grown, including, among others, *Ophiocytium, Botrydium* and *Tribonema.*

PYRRHOPHYTA (Peridinieae): Only species of *Peridinium* have been cultured. These multiply and keep exceedingly well.

PYRRHOPHYTA (Cryptophyceae): Almost every cell of the dozens of forms tested multiplied well, whereas pure cultures generally could not be obtained. *Chilomonas* and *Cyathomonas* also grew well.

CHLOROPHYTA (Volvocales): In many instances soil extract is better than soil-water cultures, e.g., for the three British species of *Volvox.* Only in soil-water cultures could be grown: *Chlamydobotrys* and *Mesostigma* which both need an additional organic ingredient. Attempts at purification in bacteria-free media failed. In acid peat with water but without soil *Carteria acidicola* John grows well. Addition of soil or soil extract prevents growth, whereas ammonium-salts up to about 0.05 per cent prolong multiplication. An exceptional case is also the larger *Chlamydomonas spharerella* (nom. prov.) which is popular for demonstration. It grows well in good light with dilute mixtures of acetate, beef extract, and peptone, but refuses to multiply in soil-water cultures. Of colorless genera, *Polytoma, Hyalogonium, Polytomella* thrive in soil-water cultures with starch or cereal grains.

CHLOROPHYTA (Zygnematales): Media have to be well chosen. *Spirogyra, Mougeotia, Zygnema, Cosmarium* and other desmids from non-peaty habitats can be grown easily with small amounts of soil. *Hyalotheca* and some others grow best in peat with water, but experience with species from acid bogs is limited.

Of larger Chlorophyta, *Cladophora, Vaucheria, Hydrodictyon, Eremosphaera,* various Chaetophorales, and a great many other forms give no difficulties.

EUGLENOPHYTA: Every species could be grown with the exception of *Euglena obtusa* Schmitz (syn. *E. limosa* Gard). which lives on periodically flooded mud flats. All the other species of *Euglena* as well as *Eutreptia, Phacus, Lepocinclis, Trachelomonas, Cryptoglena, Colacium,* and other species of the colorless genera, *Astasia, Distigma, Menoidium, Rhabdomonas, Hyalophacus, Cyclidiopsis, Entosiphon, Peranema,* and others grew well from single cells. Contrary to these results and in spite of repeated efforts relatively few Euglenophyta have been grown so far in pure culture; namely a few species of *Euglena, Trachelomonas* and *Colacium,* and apochlorotic races of *Euglena gracilis* Klebs.

CYANOPHYTA: These are developed extraordinarily well in soil-water cultures, most of them preserving their color and natural appearance for a long time, especially upon addition of ammonium-magnesium phosphate. Specis of *Spirulina,* including the rare colorless *S. albida* Kolkwitz, as well as *Beggiatoa,* multiply well.

Among the peculiar forms believed to contain symbiotic Cyanophyta as chloroplast substitutes, *Cyanophora paradoxa* Korsch. and *Glaucocystis Nostochinearum* Itz., the only ones found, could be grown in uni-algal cultures. Various Ciliata and *Spirillum volutans,* the largest species in the genus, never grown in pure culture, can also be maintained regularly.

Experience shows that the soil-water technique, although not a substitute for pure cultures, has definite advantages coupled with drawbacks. Its simple application recommends it to those phycologists who have no bacteriological training nor equipment. To the ecologist and physiologist it offers new problems but also a new road of approach.

THE ISOLATION, PURIFICATION, AND NUTRIENT SOLUTION REQUIREMENTS OF BLUE-GREEN ALGAE

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INTRODUCTION

Although some species of blue-green algae produce serious nuisance blooms and growths in reservoirs, lakes, and streams, surprisingly little is known of their exact nutritional requirements. This undoubtedly is due in part to the difficulties that have been encountered in growing many of these organisms in pure culture, a point stressed in a recent publication by Rodhe (1948). It is true that a few blue-green algae have been cultured for many years, for example, species from the family *Nostocaceae* in studies on nitrogen-fixation (Fogg, 1947), but to our knowledge the principal bloom-producers, such as *Microcystis aeruginosa* Kütz. Elenkin, *Aphanizomenon flos-aquae* (L.) Ralfs and *Gloeotrichia echinulata* (J. E. Smith) Richter, have not been successfully grown in artificial media for extended periods.

For this reason, a project was started in the botany department of the University of Wisconsin in 1947, as part of a general program of Lakes and Streams Investigations at the University, with the purpose of accumulating basic data on the nutritional requirements and physiology of blue-green algae. It was considered essential first to isolate a number of species in bacteria-free cultures, for only with pure cultures would the data obtained be specific for the algae.

This paper will report the procedures used and progress made in isolating, purifying from bacteria, and culturing representative species of these organisms. The results of experiments on

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the mineral nutrition of *Coccochloris Peniocystis* (Kütz.) Drouet and Daily also will be presented. This is the only species on which detailed data have been obtained so far.

NUTRIENT SOLUTION FOR BLUE-GREEN ALGAE CULTURE

A survey of the literature indicated that nutrient solutions had not been developed which were generally successful for the continuous culture of blue-green algae, a point also apparent from other papers presented in this symposium. Several solutions were tried which have been used extensively in investigations of green algae and diatoms, and, of these, Chu No. 10, one of the seventeen nutrient solutions published by S. P. Chu (1942), gave most promising results.

It was soon found that Chu No. 10 solution was improved as a culture medium for blue-green algae by substituting an organic source of iron, ferric citrate, for the ferric chloride specified by Chu. This improvement, due to increased iron availability, also has been reported by Rodhe (1948). Furthermore, Rodhe (l.c.) found by tests with o-phenanthroline that the addition of an equal amount of citric acid with the ferric citrate stabilized the concentration of reactive iron in the nutrient solution. This modification was adopted. Therefore, the nutrient solution used for the isolation work had the following composition in grams per liter: $Ca(NO_3)_2$, 0.04; K_2HPO_4, 0.01; $MgSO_4 \cdot 7H_2O$, 0.025; Na_2CO_3, 0.02; Na_2SiO_3, 0.025; ferric citrate, 0.003; and citric acid, 0.003. The algae have been grown in 125 ml. Erlenmeyer flasks containing 75 ml. of nutrient solution and have been kept at approximately 25°C. under continuous fluorescent light of approximately 40 foot candles intensity.

With the above modification in the iron source, Chu No. 10 solution has been a satisfactory nutrient solution for the continuous culture of twenty-four species of blue-green algae. It should not be assumed, however, that it is the optimum culture medium for any of the species. Nutritional studies with variations in the amounts of essential elements in the solution may show that modifications of Chu No. 10 will result in a faster rate and greater amount of growth of many of the algae. This is clearly indicated by the data to be presented on the nutrient requirements of *Coccochloris Peniocystis* (Kütz.) Drouet and Daily.

Uni-Algal Cultures

The lakes surrounding the city of Madison, Wisconsin, contain profuse growths of numerous species of blue-green algae, as well as green algae and diatoms, during the summer months, and various methods of isolating certain blue-greens from these waters have been attempted. The usual bacteriological techniques of streaking lake water on 2% agar plates of Chu No. 10 nutrient solution or pouring plates with small amounts of the lake water added to tubes of melted agar were unsuccessful, however, because green algae and diatoms grew rapidly and covered the agar surface before the desired blue-greens could develop.

A micromanipulator was also tried as a means of separating and removing algal cells or filaments from samples of lake water. Under microscopic observation, single cells and filaments could be drawn easily into a micropipette, but all except one species failed to grow when transferred to Chu No. 10 agar slants or solution.

In the isolation procedure that proved to be most successful, a heavy suspension of algae in lake water was collected with a plankton net at a time when the species to be isolated was predominant. Small portions of this suspension were then inoculated into a large number of flasks or tubes of Chu No. 10 solution. The growth that occurred always consisted of a mixture of species, but the cultures in which the desired organism was most abundant were selected and subcultures prepared from them. By repeated subculturing, uni-algal cultures were obtained. The fact that the desired species often rose to the surface of the nutrient solution or adhered to the sides of the flasks facilitated their separation. If the algae developed macroscopic colonies or filaments, these were picked from the lake sample or nutrient solution and washed repeatedly before subculturing to reduce the number of contaminants.

TABLE 1—Summary of results on isolation and culture of blue-green algae.

Culture No.	*Species*	*Date isolated*	*Approx. days for maximum growth*	*Bacteria-free cultures* (X)
1001	*Phormidium minnesotense* (Tild.) Drouet	9– 4–47	12 to 15	
1013	*Nostoc muscorum* Ag.	not available	15 to 18	X
1017	*Plectonema Nostocorum* Gom.	9– 4–48	15 to 18	X
1018	*Calothrix parietina* (Näg.) Thur.	10–19–48	40 to 50	X
1019	*Phormidium tenue* Gom.	9–13–48	15 to 18	X
1020	*Coccochloris Peniocystis* (Kütz.) Drouet and Daily [*Gloeothece linearis* Näg.]	10–27–48	10 to 12	X
1021	*Diplocystis incerta* (Lemm.) Drouet and Daily [*Microcystis incerta* Lemm.]	11–19–48	10 to 12	
1026	*Lyngbya Birgei* G. M. Smith	10–19–48	40 to 50	
1029	*Anacystis marina* (Hansg.) Drouet and Daily [*Aphanocapsa marina* Hansg.]	11–15–48	12 to 15	
1032	*Aphanizomenon flos-aquae* (L.) Ralfs	11–28–48	18 to 20	X
1034	*Lyngbya versicolor* Gom.	12–14–48	18 to 20	
1036	*Diplocystis aeruginosa* (Kütz.) Trevis [*Microcystis aeruginosa* Kütz.]	9–27–48	10 to 12	X
1038	*Anabaena circinalis* (Kütz.) Rab.	11– 3–48	20 to 25	
1041	*Gloeocapsa membranina* (Menegh.) Drouet and Daily	11–12–48	30 to 35	X
1042	*Gloeocapsa dimidiata* (Kütz.) Drouet and Daily [*Chroococcus turgidus* (Kütz.) Näg.]	not available	30 to 35	X
1043	*Phormidium autumnale* Gom.	12–10–48	20 to 25	
1044	*Arthrospira Jenneri* Stiz.	1–25–49	20 to 25	
1046	*Amphithrix janthina* Born. and Flah.	1–29–49	30 to 35	
1047	*Oscillatoria princeps* Vauch.	1–29–49	35 to 40	
1048	*Cylindrospermum licheniforme* Kütz.	3–28–49	35 to 40	
1049	*Nostoc commune* Vauch.	3–28–49	35 to 40	
1051	*Gloeocapsa alpicola* (Lyngb.) Born.	10– 6–49	25 to 30	
1052	*Gloeotrichia echinulata* (J. E. Smith) P. Richt.	8– 9–49	15 to 18	

Twenty-two species of blue-green algae have been isolated by the above procedure. All but one of these have been identified by Dr. Francis Drouet of the Chicago Natural History Museum and are listed in Table 1. For some cultures (1020, 1021, 1029, 1036, and 1042), the more familiar equivalents are included with the names assigned to them by Dr. Drouet. The new names given these species have been published by Drouet and Daily (1948) and are to be fully explained and discussed in a revision of the *Chroococcaceae* they are now preparing for publication. The number assigned to each culture is the number by which that particular species is identified in this laboratory, and that by which it will be designated as a permanent specimen in the Chicago Natural History Museum collection.

Two species (1013 and 1042) which have been obtained from Dr. Robert Emerson of the University of Illinois are also included in Table 1. In addition, this table gives, where available, the date on which each species was isolated and the approximate time required for maximum growth to develop after inoculation into flasks of Chu No. 10 solution.

The time required for maximum growth varies widely with the species. For example, *Coccochloris Peniocystis* (Kütz.) Drouet and Daily, *Diplocystis incerta* (Lemm.) Drouet and Daily, and *Diplocystis aeruginosa* (Kütz.) Trevis (cultures 1020, 1021, and 1036) reach a maximum in only 10 to 12 days, whereas *Calothrix parietina* (Näg.) Thur. and *Lyngbya Birgei* G. M. Smith (cultures 1018 and 1026) require 40 to 50 days. However, as mentioned previously, these relative rates of growth can be considered specific only with Chu No. 10 solution as the culture medium.

BACTERIA-FREE CULTURES

Bacteria normally penetrate and live in the gelatinous sheaths which surround the cells and filaments of blue-green algae so that their removal by plating, washing, or dilution is very tedious and difficult, if not impossible. For this reason, ultra-violet irradiation, employed by Allison, et al. (1930, 1937) to obtain a bacteria-free culture of *Nostoc,* was used to kill the bacteria associated with the algae.

A dilute algal suspension was placed in a quartz-windowed chamber and irradiated for 20 to 30 minutes with 2750 Ångström ultraviolet light from a quartz-jacketed mercury vapor lamp. The suspension was agitated by continuous stirring during the irradiation period. Samples were taken at five-minute intervals, and a large number of dilution cultures was prepared from each. Subcultures usually failed to grow when taken from samples with long exposures and were usually contaminated when taken from samples with short exposures. Certain subcultures, however, particularly from samples with intermediate exposures, were found to be free of bacteria. Obviously, not all the bacteria were killed in the irradiation chamber, nor did all the algae survive. Rather, the algae were slightly more resistant to the ultra-violet than the bacteria, so that by sufficient dilution of some samples it was possible to isolate a few cells or algal filaments free of bacteria.

After preliminary tests in a yeast-dextrose medium had indicated that the algae were free of bacteria, they were further checked for purity on sodium caseinate agar to detect aerobic bacteria (other than nitrogen-fixers) and Actinomycetes (Stark and McCoy, 1938), in both Ashby's and Burk's nitrogen-free media for *Azobacter* (Wilson and Knight, 1947), and in Brewer's medium for anaerobes (Brewer, 1940).

Nine of the species in Table 1 have been freed of bacteria by the above procedure, and a tenth pure culture has not been identified as yet. Although these cultures include a variety of growth forms, the majority were purified by one or two treatments with ultra-violet light. The method, therefore, is relatively simple and should be satisfactory for purification of the remaining species.

MINERAL NUTRITION OF COCCOCHLORIS PENIOCYSTIS

Experimental Procedure

Because *Coccochloris Peniocystis* (Kütz.) Drouet and Daily [*Gloeothece linearis* Nág.] was the first rapid-growing, identified species obtained in pure culture, it was selected for the initial detailed mineral nutrition studies. Chu No. 10 solution was not a suitable culture medium for this work, as each of several of the salts used provides more than one of the elements essential for plant growth, thus making it impossible to study the effect of variations in the concentration of a single element. For this reason, the nutrient solution presented in Table 2 was developed. Whereas the concentrations of the essential elements are the same as in Chu's No. 10 solution, the cations are present as chloride salts and the anions as sodium salts which permit independent variation of each essential element. The sodium carbonate and sodium silicate present in Chu No. 10, ferric citrate - citric acid as an iron source, and Hoagland's A-Z solution of minor elements are included.

TABLE 2—Composition of basic culture solutions used for mineral nutrition experiments with *Coccochloris Peniocystis.*

Compound	*Grams per liter*	*Ppm. of essential elements*	
$NaNO_3$	0.0413	N	6.8
Na_2HPO_4	0.0082	P	1.8
KCl	0.0086	K	4.5
$MgCl_2\ 6H_2O$	0.0209	Mg	2.5
Na_2SO_4	0.0146	S	3.3
$CaCl_2\ 2H_2O$	0.0359	Ca	9.8
Ferric citrate	0.003	Fe	0.56
Citric acid	0.003		
Na_2CO_3	0.02		
Na_2SiO_3	0.025		

Hoagland's A to Z solution is added to the culture solution at 1/25 the strength specified for higher plants.

The culture vessels were 500 ml. Erlenmeyer flasks containing 200 ml. of the appropriate nutrient solution. Pyrex glassware and pyrex glass - distilled water were used for all solutions and culture media. Four cultures were prepared for each treatment, and, after autoclaving, were inoculated with one milliliter of an actively growing suspension of bacteria-free *Coccochloris Peniocystis.* In all operations, precautions were taken to exclude bacteria from the flasks. The cultures were illuminated continuously with fluorescent light of approximately 50 foot candles intensity and kept at approximately 25° C.

The determination of the amount of growth in cultures of *Coccochloris Peniocystis* proved to be a difficult problem. The cells are so small that they readily pass through coarse filters, yet, probably because of their gelatinous sheaths, clog fine filters almost immediately. Colorimetric and turbidimetric procedures were rejected because of errors introduced by variations in the color and degree of flocculation of the algal growths with modifications of the culture medium. It was even difficult to separate the cells from the nutrient solution by centrifugation, because part of the growth remained in suspension or was concentrated at the surface of the solution. The addition of 100 ppm. $Al_2(SO_4)_3$ to the culture medium at the time of harvest and the lowering of the pH to 4.5, however, flocculated the cells so that after centrifuging for twelve minutes at 2700 RPM they collected in a tight mat at the bottom of the tube. In most cases, the 100 ppm. of $Al_2(SO_4)_3$ lowered the pH to the desired level. If not, it was adjusted to 4.5 with dilute HCl. The centrifuged cells were washed once in distilled water, rinsed into a tared weighing bottle, and dried to constant weight at 63° C. The yields from two flasks were placed in one tared weighing bottle to provide sufficient material for an accurate dry weight determination.

pH Experiment

Because it would be difficult to buffer the pH of cultures of *Coccochloris Peniocystis* at unit intervals between 5.0 and 11.0 and still not influence the growth of the algae in other ways, the effect of variations of pH on the growth of this organism was determined by frequent adjustments with dilute HCl and NaOH. The Na_2CO_3 usually included in the culture medium was omitted in this experiment to facilitate maintenance of the solutions at the desired pH levels. The pH of the cultures in which there was little or no growth was adjusted every twenty-four hours. In the cultures in which considerable growth developed, however, adjustments were made every twelve hours so that the pH was maintained within half a unit of the specified values. In these cases it was impractical to maintain sterile conditions, but this is the only experiment to be reported in which the cultures were not kept free of contaminants.

TABLE 3—Effect of pH of the culture medium and the presence of Na_2CO_3 and Na_2SiO_3 on the growth of *Coccochloris Peniocystis.* Cultures A to H inoculated on Feb. 20, 1950 and harvested on March 1, 1950. Cultures H, I, and J inoculated on Nov. 9, 1949 and harvested on Nov. 18, 1949.

Culture	*Treatment*	*Oven-dry wt. of algae* (mg. per 400 ml. solution)
A	Basic solution with 3 × NO_3 but no Na_2CO_3 pH adjusted daily to 5.0.	no growth
B	As A, pH adjusted daily to 6.0.	no growth
C	As A, pH adjusted daily to 7.0.	4.9
D	As A, pH adjusted daily to 8.0.	12.7
E	As A, pH adjusted daily to 9.0.	40.8
F	As A, pH adjusted daily to 10.0.	69.2
G	As A, pH adjusted daily to 11.0.	48.4
H	Basic solution with 3 × NO_3, and pH not adjusted. Initial pH, 8.4; final pH, 10.3.	27.2
I	As H, except both Na_2CO_3 and Na_2SiO_3 omitted. pH adjusted daily to that of treatment H on same day.	22.1
J	As I, but pH not adjusted. Initial pH 6.7; final pH 7.5.	3.9

The results presented in Table 3 show that *Coccochloris Peniocystis* requires an unusually high pH for maximum growth. There was no growth in cultures maintained at pH 5.0 and 6.0, and only very slight growth at 7.0 and 8.0. Growth increased considerably at pH 9.0, reached a maximum at pH 10.0, then declined again at pH 11.0.

If other blue-green algae require as high a pH for maximum growth as *Coccochloris Peniocystis,* nutrient solutions suitable for their culture must contain not only the proper concentrations of the essential elements, but salts must be selected that will maintain the pH of the medium above 8.0. Possibly, therefore, the alkaline buffering provided by the Na_2CO_3 and Na_2SiO_3 may account for the general suitability of Chu No. 10 solution for the culture of blue-green algae. This is indicated by Treatments H, I, and J of Table 3. Treatment H is the basic solution with 3 times $NaNO_3$. In Treatment I, Na_2CO_3 and Na_2SiO_3 were omitted from the nutrient solution, but the pH of the solutions was adjusted daily to those of Treatment H in which the pH gradually increased from 8.3 when inoculated to 10.4 at the time of harvest. There was only a slight difference in the yields from these two treatments. In Treatment J, however, in which Na_2CO_3 and Na_2SiO_3 were omitted from the basic solution and the pH was left unadjusted, there was almost no growth. Similar results have been obtained with *Nostoc muscorum* Kütz. which show that Na_2CO_3 and Na_2SiO_3 need not be added to the culture medium to provide essential elements, but that their favorable effect for culturing at least these two species is related to their alkaline buffering capacity.

CONCENTRATIONS OF ESSENTIAL ELEMENTS NECESSARY FOR MAXIMUM GROWTH

Before starting experiments to determine the effects of variations in the concentration of a single element on the growth of *Coccochloris Peniocystis,* it was necessary to ascertain if the basic solution, as presented in Table 2, contained at least optimal amounts of all the essential elements for the maximum growth of this organism, so that only the elements under investigation could become limiting. Consequently, culture solutions were prepared in which all the constituents were present at 2 times or 3 times the concentrations in the basic solution. Although heavy precipitates formed in these solutions and made accurate dry-weight determinations impossible, it was evident from visual observation that both treatments produced more growth than the basic solution. Systematic lowering of the concentration of one element at a time from triple to double the concentration, and to the level of the basic solution showed that higher nitrate concentrations were solely responsible for the increases in growth. The difference in yields between double and triple strength nitrate was very slight, but, in order that yields would not be limited by a deficiency of nitrate, in all subsequent mineral nutrition experiments the nitrate concentration in the culture solution was 3 times that of the basic solution.

TABLE 4—Effect of varying the concentration of nitrate in the nutrient solution on the growth of *Coccochloris Peniocystis.* Cultures inoculated on Nov. 10, 1949 and harvested on Nov. 17, 1949 and Nov. 22, 1949.

		Oven-dry weight of algae (mg. per 400 ml. solution) *after*		pH *of solution after 12 days*
Culture	NO_3 *concentration*	7 days	12 days	
A	3 ×	35.0	73.1	10.8
B	2 ×	32.1	67.4	10.4
C	× (6.8 ppm. N)	34.8	59.6	9.8
D	× /2	26.6	25.2	8.1
E	× /4	11.2	14.3	8.0
F	× /10	4.7	5.7	8.0
G	× /20	2.3	3.9	8.0
H	× /40	2.1	4.4	8.0

VARYING THE CONCENTRATION OF THE ESSENTIAL ELEMENTS

Table 4 shows the dry weights of *Coccochloris Peniocystis* obtained after seven and twelve days of growth in solutions varying in nitrate concentration from 3 times to 1/40th the concentration in the basic solution. Two of the four cultures of each treatment were harvested after seven days of growth and the remaining two after twelve days to avoid any error that might result if cultures containing suboptimal concentrations of nitrate reached maximum growth in a relatively short time, then decreased in dry weight before the cultures with higher nitrate concentrations had reached their maxima. Such decreases in weight did not occur, however, for in cultures D through H where growth was limited by low nitrate and reached a maximum in seven days, the dry weights did not decrease during the next five days.

The dry weight determinations after twelve days of growth show that for maximum yields of *Coccochloris Peniocystis* the nutrient solution must contain a relatively high concentration of nitrate. The 5.7 mg. increase in dry weight produced by Treatment A over Treatment B is probably not significant. However, the 13.5 mg. difference between Treatments A and C is significant, so that at least 13.6 ppm. of nitrogen are necessary for maximum growth of this organism under the conditions of this experiment. This was substantiated by the appearance of the cultures at the twelve-day harvest, for those of Treatments A and B were still a dark blue-green color while cultures of Treatment C had turned a yellowish-green. Furthermore, chemical tests showed that nitrate was completely absent from cultures of Treatments B and C, yet approximately 6.0 ppm. of nitrate nitrogen remained in the cultures of Treatment A.

Table 4 also shows the pH of the culture solutions at the twelve-day harvest. In this and other experiments, the pH of the culture solutions was approximately 8.0 when inoculated, and in the solutions with the heaviest growths it gradually increased to 10.5 and 11.0 after twelve to fourteen days. From the results of the pH experiment, this alkaline shift would seem to be highly desirable. It was probably due to removal by the algae of bicarbonate ions present in the equilibrium mixture produced by Na_2CO_3 in the culture solution and CO_2 from the atmosphere as well as to rapid utilization of the nitrate ion.

Table 5 presents the results of additional experiments on the effects of varying individually the concentrations of phosphorus, sulfur, potassium, calcium, magnesium, and iron on the growth of *Coccochloris Peniocystis.* The amount of growth in two of the four cultures of each treatment was determined after approximately seven days of growth and in the remainder after fourteen days. As in the nitrate experiment, however, a single harvest at fourteen days would have been sufficient, for, although cultures containing suboptimal concentrations of an element usually produced maximum growth within seven days, they did not decrease significantly in weight during the second week. For ease of comparison, the yield from each treatment is reported as a percentage of that in the basic solution, Treatment C, rather than as weight of dried cells.

TABLE 5—Summary of experiments on the mineral nutrient requirements of *Coccochloris Peniocystis.*

Concentration of element varied		*Growth as per cent of that in the basic soln.* (Treat. C)						
		NO_3	PO_4	SO_4	K	Mg	Ca	Fe
A	3 × basic soln.	123	103	94	92	98	93	—
B	2 × " "	113	102	104	99	108	97	92
C	× " "	100	100	100	100	100	100	100
D	×/2 " "	42	99	102	94	93	106	—
E	×/4 " "	24	101	101	88	96	105	95
F	×/10 " "	10	79	75	61	94	107	100
G	×/20 " "	7	53	54	43	91	101	94
H	×/40 " "	7	35	37	39	70	112	87
I	None	—	12	20	25	17	110	60
Highest yield (mg.)		A 73.1	A 122.4	B 111.1	C 120.4	B 121.9	H 119.3	F 115.2

The nitrate concentration for all experiments, except that in which nitrate was varied, was 3 × that in the basic solution.

The concentration of phosphate required for maximum growth was surprisingly low when compared to nitrate. The basic solution, Treatment C, contained only 1.8 ppm. phosphorus, yet maximum growth was still produced at one-fourth this concentration or at 0.45 ppm. The minimum nitrogen concentration for maximum growth was 13.6 ppm. nitrogen, thus, these results indicate that maximum algal growth could be produced with a N/P ratio in the nutrient solution of at least 30/1.

As with phosphate, the sulfate concentration could be lowered to one-fourth that in the basic medium solution without decreasing growth. But the basic solution contained 3.3 ppm. sulfur and only 1.8 ppm. phosphorus so that for maximum growth this organism required almost twice as much sulfur as phosphorus in the medium.

Of the cations, potassium was required in the largest amounts, for growth decreased when the potassium concentration was less than 2.25 ppm. or one-half the amount in the basic solution. The magnesium concentration could be lowered to 0.13 ppm., which is one-twentieth the concentration in Treatment C, without significantly decreasing the amount of growth. Inasmuch as there is usually a high calcium concentration in the eutrophic lakes where blue-green algae are most abundant, it seems surprising that the growth of *Coccochloris Peniocystis* did not decrease when calcium salts were omitted from the nutrient solution. This does not mean that calcium was completely absent, for a small amount was introduced with the one milliliter inoculum and was present as impurities in other than calcium compounds added to the culture medium.

The results in Table 5 also show that, with ferric citrate as the iron source, the iron concentration could be much lower than the 0.56 ppm. in the basic solution. In fact, growth did not decrease until the iron concentration was less than 0.03 ppm., or one-twentieth the amount present in Treatment C.

The above results give the minimum concentrations of individual elements required to produce optimal yields when each is varied singly. There is reason to believe, however, that a nutrient solution containing only these minimum concentrations of the nutrients might not produce maximum yields. For example, it has been established that for higher plants there is an optimum range of osmotic or total salt concentration. Furthermore, both the extent of uptake and the actual requirement for each ion will be determined in part by the concentrations of other ions in the nutrient solution. From the data in Table 5, a solution containing the minimum concentration of each element for the optimum growth of *Coccochloris Peniocystis* would have the following composition in ppm.: nitrogen, 13.6; phosphorus, 0.45; sulfur, 0.83; potassium, 2.25; magnesium, 0.13; iron, 0.03; and only traces of calcium derived from the inoculum and from impurities in the nutrients. When such a solution was tested, the yield was 91.5 mg. as compared with 109.7 mg. for the basic solution with triple nitrate concentration, and with double the minimum concentration of each element the yield was 118.2 mg. It is evident, therefore, that, whereas some adjustment of the nutrient solution may be necessary for determination of the exact minimum requirements for maximum growth, the procedure followed above gives a very close indication of the actual proportions in which the elements are required under the conditions of these experiments.

Comparison of Ferric Chloride and Ferric Citrate as an Iron Source

When the present work on the isolation of the blue-green algae was first started, a stock solution of ferric chloride was prepared and used continuously as a source of iron in Chu No. 10 solution. It was soon found, however, that the algae made better growth with fresh ferric citrate as an iron source. It was later thought that possibly the ferric chloride solution became unsatisfactory upon standing due to a slow conversion of the iron into a form unavailable to the algae. Therefore, the experiment reported in Table 6 was carried out to compare freshly dissolved ferric chloride and ferric citrate plus citric acid and a twenty-month-old ferric chloride solution as sources of iron for *Coccochloris Peniocystis.*

The data in Table 6 show that at high concentrations of iron, 1.12, 0.56, and even 0.14 ppm., the fresh ferric chloride and ferric citrate are equally effective. At the lower concentrations, 0.06 and 0.03 ppm., however, the ferric citrate produced greater yields. At all concentrations, the twenty-month-old ferric chloride solution produced less growth than the fresh ferric chloride or ferric citrate, showing that a stock solution of ferric chloride cannot be used indefinitely as an iron source for algal culture. In fact, the old ferric chloride solution was inhibitory at high concentrations, for growth increased as the iron concentration in the culture solution was reduced. This effect may have been caused by gradual conversion of the iron to Fe_2O_3 which when added to the culture solution precipitated the phosphate in an unavailable form.

Table 6—Comparison of ferric citrate and ferric chloride as a source of iron for *Coccochloris Peniocystis.* Cultures inoculated on Dec. 3, 1949 and harvested Dec. 16, 1949.

	Oven-dry weight of algae (mg.) in 400 ml. solution		
	Ferric citrate solution	*Ferric chloride solution*	
Concentration of Fe	*3 days old*	*3 days old*	*20 months old*
1.12 ppm.	105.5	105.7	47.9
0.56 ppm.	114.7	111.1	44.1
0.14 ppm.	109.5	99.1	48.0
0.06 ppm.	115.2	81.2	70.6
0.03 ppm.	108.1	75.0	70.4

DISCUSSION

The results presented in this paper show that many species of blue-green algae, including the bloom-producers, *Microcystis aeruginosa* Kütz. Elenkin, *Gloeotrichia echinulata* (J. E. Smith) Richter, and *Aphanizomenon flos-aquae* (L.) Ralfs, can be cultured continuously in inorganic solutions of completely known composition. This is significant in view of the references in the literature to the possible importance of organic substances for the growth and successful culturing of blue-green algae (Hutchinson, 1944), (Pearsall, 1932), (Rodhe, 1948). As a specific example, Rodhe (l.c.), who obtained some growth of *Gloeotrichia* in a culture of soil and lake water, but no growth after the soil and water had been heated, and no growth in nutrient solutions of known composition states, "It seems that *Gloeotrichia* requires for its growth access to one or several organic substances which are not thermostable." Also, Pringsheim (1946) found that soil-and-water cultures as well as various extracts and organic materials were useful for culturing many algae. Even in the early work on the present project, when the first attempts to culture some species, including *Microsystis aeruginosa* Kütz. Elenkin and *Gloeotrichia echinulata* (J. E. Smith) Richter, were unsuccessful, it was thought that the nutrient medium perhaps lacked some essential organic constituents, and extracts of leaf-mould, soil, and other organic materials were added to the culture solutions. The extracts never meant the difference, however, between growth and no growth. They did influence the rate of growth to a slight extent, but not to a greater degree than could be duplicated by modifications of the inorganic solutions. The difficulties encountered in culturing some species apparently were due to an unsatisfactory physiological condition of the algae obtained in the first lake water samples, for, in all cases, persistent sampling of different parts of the lakes provided cells that grew satisfactorily in inorganic media. It may be that further investigations will reveal organic substances which will stimulate growth beyond that produced in inorganic solutions. The fact that twenty-four species of blue-green algae are in continuous culture in one inorganic solution, however, strongly indicates that as a group they do not require organic substances or unknown factors for normal growth.

The mineral nutrition experiments with *Coccochloris Peniocystis* (Kütz.) Drouet and Daily show the relative amounts of the major essential elements required for maximum growth and illustrate a method by which quantitative information on the nutrition of other blue-green algae may be obtained quite easily once the organisms are obtained in pure culture so that they can be grown under controlled conditions. From a practical standpoint, probably the most important result from these experiments was that this organism requires such a high level (13.6 ppm.) of nitrate nitrogen as compared to phosphorus (0.45 ppm.) to produce maximum growth. If bloom-producing species have similar requirements, and experiments now in progress indicate that this is true for *Microcystis aeruginosa,* nitrogen supply must be an important factor in controlling the growth of blue-green algae under natural conditions. Furthermore, the high nitrate requirement together with the direct relationship between yield and added nitrate over a wide range indicate that *Coccochloris Peniocystis* in all probability does not fix atmospheric nitrogen as some blue-green algae have been shown to do.

A question might be raised regarding the applicability of results obtained in culture flasks in the laboratory to conditions existing in lakes and streams. The results with *Coccochloris Peniocystis* indicate that the laboratory data cannot be applied directly to field conditions. For example, the maximum inorganic nitrogen concentration in the lakes and streams near Madison, Wisconsin, where blue-green algae grow in great abundance is far below the 13.6 ppm. necessary for maximum growth in the laboratory experiments. Not only the concentration of the essential elements, but also the volume of the solution from which the algae can extract them must be important in determining the suitability of a particular environment for the growth of blue-green algae. Nevertheless, data obtained in the laboratory on the qualitative growth requirements of blue-green algae and the relative proportions of the essential elements necessary for maximum growth should be of considerable value in determining the requirements for optimum growth of these organisms in lakes and streams.

Acknowledgements

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THE CULTURE OF ALGAE FOR PHYSIOLOGICAL RESEARCH[3]

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The culturing of algae for physiological studies has called forth a number of different techniques developed according to the objectives of the work. Most physiological work on the algae has been done with unicellular Chlorophyta such as *Chlorella, Scenedesmus,* and *Stichococcus*. My remarks therefore will be concerned principally with the culturing of forms such as these.

One common objective is to understand nutrition or metabolism of an alga in terms of its growth. This constitutes the simplest and most productive starting point for any physiological work. Experimental design must afford illumination, provide a carbon source (usually CO_2), maintain constant temperature, and include a means of estimating growth. In some cases growth experiments have utilized cotton-plugged flasks, but if CO_2 is the carbon source, such procedure is highly questionable. One very likely may be studying the physical characteristics of the vessel in determining CO_2 diffusion rather than the characteristics of the algae. Oesterlind (1949) has introduced a technique of providing carbon dioxide as bicarbonate in the media and using vessels sealed with rubber stoppers. More common procedure (e.g. Warburg, 1919; Pratt, 1940; Winokur, 1948) has been to use flasks of a design essentially similar to that shown in Figure 1. A 250 cc. or 500 cc. Erlenmeyer is provided with inlet and outlet tubes for aeration and a rubber cap which can be used to cover the cotton plug after inoculation. A mixture of 5% CO_2

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[3] This paper is based, in part, on work performed under contract N8-onr-78000 with the Office of Naval Research.

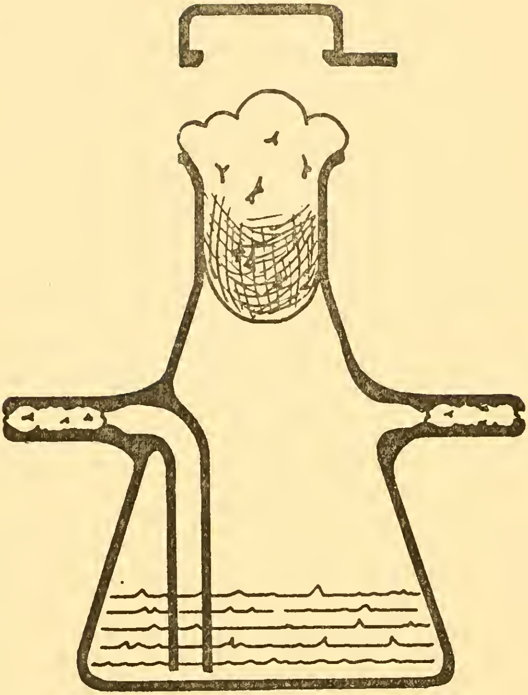

FIG. 1—Flask for algal culture with inlet and outlet aeration tubes.

in air is commonly used for aeration. The gas may be conserved by placing several flasks in series as is made possible by their design. A general problem of this design is that even with high (and expensive) rates of aeration the algae tend to settle out; some mechanical means of agitation, therefore, becomes necessary. In conjunction with the use of such vessels it has been common practice to estimate growth in terms of cell number as determined by hemocytometer counts, in terms of cell-volume as determined by centrifuging in hematocrits, by weighing cell crops, or by turbidimetric readings.

It is our experience that the modified Erlenmeyer flask is a poor compromise between elegance and simplicity and we have preferred to make modifications in each direction. Our simplest apparatus is built around the use of test tubes which fit into a photoelectric colorimeter so that growth can be followed turbidimetrically directly in the culture vessel. The apparatus is illustrated in Figure 2.

Test tubes 21.5 mm. by 175 mm. are used as culture vessels. Agitation, and carbon dioxide supply are provided by passing in an air : carbon dioxide mixture (95: 5) through a bubbling tube. The bubbling tube is constricted about 50 mm. below its upper end in order to hold a small cotton plug which is inserted into the test tube through a larger cotton plug protected by a gauze wrapping. The 5% carbon dioxide gas mixture is prepared by allowing compressed air and compressed carbon dioxide under constant pressure to pass through orifices so chosen as to obtain the correct mixture. Flow rates of about 50 cc. per sec. for air and 2.5 cc. per sec. for carbon dioxide are indicated by Venturi flow gauges. The gas mixture is held at about 2 lb. per sq. in. pressure by allowing some of the excess gas to escape against a head of water. Manifolds providing 24 outlets per bath have been constructed from brass tubing and inexpensive needle valves (Imperial No. 103). The water baths are built from ½ in. by 6 in. aluminum stock, channelled to allow fitting of plate glass sides; they are 22 in. long by 7½ in. deep and accommodate 24 tubes each. Thermostated 25° C. water is pumped through the baths continuously. Illumination at a level of about 600 f.c. is provided by two banks of four 60-Watt tungsten lamps, one bank on each side. Subsequent work has shown that two 20-Watt fluorescent lamps on each side also provide adequate illumination with less heating.

The test tubes easily accommodate 10 ml. of culture medium which may be sterilized by autoclaving. In short-time growth experiments, however, it is often possible to dispense with sterile precautions. Growth is estimated as optical density in the Evelyn photoelectric colorimeter using a 600 mu. filter, the bubbling tube being raised about an inch during the reading. For any given suspension of *Chlorella pyrenoidosa* Link the optical density is proportional to population up to a density of about 1.0 which corresponds approximately to 2.0 cmm. cells per ml. or

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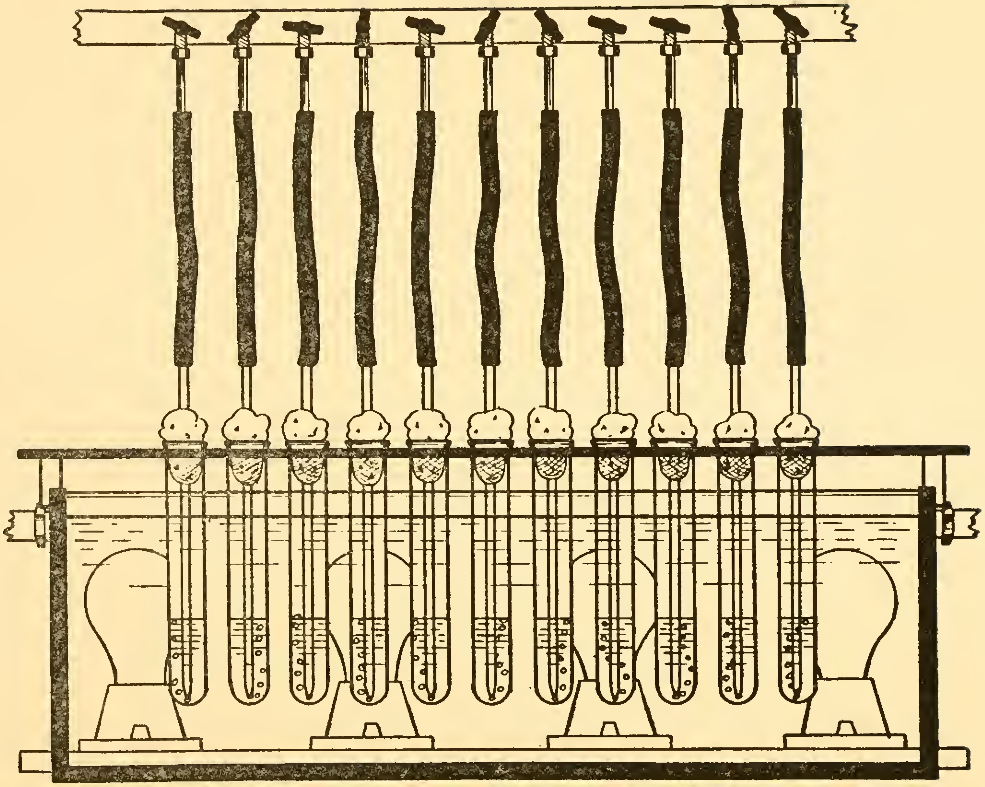

Fig. 2—Test tube cultures for turbidimetric growth studies.

50×10^6 cells per ml. It is likely that for cells grown under different conditions the optical density is not always the same function of the population. In any event the method provides a rapid means of following growth with a precision adequate for many problems. We are now using this technique as a screening method to find out some of the more obvious nutritional characteristics for a number of different algal species. Techniques similar in principle, but differing in detail, have been reported by Rodhe (1948) and Österlind (1949).

A second special design (Myers and Johnston, 1949) is a modification of the so-called roll culture often used in microbiological work. A ten-liter serum bottle containing 1000 ml. of algal suspension was used as the culture vessel. Here the objective was to obtain extensive data on one culture, in particular to study metabolism during growth. It was possible to study the gas exchange by gas analysis and to make chemical analyses on the culture medium and on the cells produced. By such study it was possible to show that *Chlorella pyrenoidosa* Link excretes only a very small amount of organic matter. About 95% of the carbon and nitrogen used up during photosynthetic growth could be recovered in the cells produced.

A third design (Myers and Clark, 1944) we have called a continuous culture apparatus. The objective here can best be explained with reference to the characteristic growth curve of a microorganism. This is commonly interpreted as describing growth under some standard or specified cconditions. Actually, however, conditions in the cellular environment do not remain constant throughout the course of the growth curve. In an algal culture increased numbers of cells cause a mutual shading with a decrease in the effective light intensity per cell. Nitrate is rapidly taken up by the cells with attendant increase in pH. Fortunately all such variables are functions of the population so that if we can hold the population constant by diluting the culture as fast as it grows, then we can also hold constant all the variables which normally accompany the growth curve.

The central glass chamber is essentially a vertical doubly-jacketed condenser built of three concentric glass tubes. Thermostated water flows through the outer jacket. The algal suspension is contained in the inner annulus which is only about 5 mm. thick. Aeration is provided through a cotton filter and in the very thin annular chamber it also produces good agitation of the suspension. Samples of the suspension are harvested as needed; the suspension is never completely harvested, however, some always being left as an inoculum. Automatic dilution is accomplished by a photometric device. As the culture grows, the increased number of cells cuts down the illumination on a photocell. An off-balance current is developed in the photocell circuit, causing a relay to open a solenoid valve and allow fresh media to flow in, diluting the culture back to a photometric balance. The result is that the culture is maintained at a point or within a very short segment of the growth curve.

The continuous culture apparatus provides the very great advantage that the effects of environmental conditions may be examined by introducing only a single variable at a time. Rate of growth is readily determined by leaving a known amount of inoculum at the end of each harvesting period and determining the amount of the harvest. In addition a daily harvest of from 25 mg. to 250 mg. of dry weight per day provides adequate quantities for most types of physiological work. The method has the disadvantage of requiring a considerable overhead of man-hours for maintenance. It has presented so many advantages, however, that we customarily maintain three units in operation.

A second general objective in culture has been the production of cellular material for metabolic studies or for chemical analysis. Experimental design here depends primarily upon the quantities of material needed. The most common procedure has been to use flasks such as those of Figure 1. By a regimen of harvesting a culture always at some one point on its growth curve, fairly reproducible material can be obtained. Our procedure, wherever possible, is to use the continuous culture apparatus which produces highly uniform experimental material day after day.

When very large amounts of algae are needed, as for chemical analysis, the quantity requirements become the deciding factor in design. For investigation of photosynthesis by use of radioactive carbon Fager (1949) has developed an aquarium-like tank one meter high by one meter wide by 10 cm. thick. Illumination by banks of fluorescent lamps allows a production of a pound of *Scenedesmus* per week. Another development for quantity production is that of Ketchum and Redfield (1938, 1949) as modified by Scott (1943). A five gallon carboy is adapted as a culture vessel. A similar development is that of Spoehr and co-workers (1949a) who used five-gallon carboys under daylight illumination in a greenhouse. An extension of culture methods to large scale algal farming is being explored by Paul Cook of the Stanford Research Institute, who will describe in another paper of this symposium his experience in development of really large scale mass culture.

Finally, brief comment well may be made on the problems of selection of culture media. Innumerable formulae are available in the literature and have been variously selected by different laboratories in the fine tradition of ancestor worship. It is our experience that the problem of culture media may be broken down into a series of specific choices with respect to (1) concentration of the major salts, (2) pH, (3) nitrogen source, (4) the provision of iron and other micro-elements, (5) the provision of organic materials or growth factors for certain forms.

I hope that I have not implied in any way that culture methods have become cut and dried. Actually, most of the methods which I have described have been developed for hardy and easily grown forms such as *Chlorella.* A great deal of ingenuity needs to be applied to the culture of a number of algae which should prove highly interesting for physiological work.

LARGE-SCALE CULTURE OF CHLORELLA

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INTRODUCTION

Scientists recently have been studying the simplest photosynthetic organisms, unicellular green algae, in their investigations of the fundamentals of photosynthesis. Drs. H. A. Spoehr and H. W. Milner of the Carnegie Institution of Washington's Division of Plant Biology at Stanford University, began such a study over eight years ago, using the common single-celled alga, *Chlorella pyrenoidosa* Link. They have shown (1949) that the chemical composition of the alga can be altered by changes in the environmental conditions under which it is grown. They produced *Chlorella* which had a protein content of over 50% by maintaining a fixed nitrogen concentration above a critical value in their culture medium. When the alga was grown in media containing less than the critical value of .001 mol fixed nitrogen-content, reproduction soon stopped and lipid was synthesized up to as high as 85% of the dry weight. In this work considerable quantities of *Chlorella* were grown and it was apparent that the rate of growth was rapid. The possibility of the large-scale growth of *Chlorella* was seen, and some calculations were made to determine what quantities of substances could be produced were the plants cultured on a given area of land.

The Research Corporation of New York was familiar with the work of Spoehr and Milner and believed a survey should be made to investigate the feasibility of making practical applications of the findings of *Chlorella* studies within the next few decades. The Stanford Research Institute was engaged to make the survey. The assignment was to analyze existing knowledge derived from laboratory studies of *Chlorella,* to consider the methods and problems involved in developing a large-scale process, and, in the event that the results were promising, to make recommendations concerning the additional work necessary to pursue the problem intelligently.

Stanford Research Institute Survey

The basic problem was to discover whether it might be feasible as well as practical to grow large quantities of algae in a controlled system. This broad problem requires detailed answers to several specific questions: 1) Is it technically possible to grow algae in large quantities in any controlled system? 2) If so, what type of system should be used? 3) What are the optimum conditions for maximum growth? 4) How valuable is the material produced? 5) What is the cost of producing large amounts of *Chlorella?*

During the suvey it became apparent that by using a simple continuous system, the mass growth of *Chlorella* might be not only feasible but actually practical today. With a few exceptions, however, all data were based on cultures in a batch system in small vessels wherein several important conditions were allowed to vary. Because of the conditions under which the experiments were made, it was difficult to translate the data to a continuous large-scale basis. In spite of this, upon analysis of the data, the large-scale culture of *Chlorella* appeared promising. Many interpretations needed carefully controlled experiments to prove their validity, however, and further data had to be obtained for more accurate engineering studies.

The value and possible use of the final product also was unknown, but an organism with a protein content of over 50% or with lipid content as high as 85% would certainly have some nutritive or chemical value as a raw material. No price could be put on its actual value at first. The high price and relative shortage of protein in the world, in addition to the fact that high-protein *Chlorella* grows more rapidly than high-fat *Chlorella,* threw a particularly favorable light on the outlook for growing this plant as a source of protein.

Certain raw materials are essential for growth. Carbon dioxide supplies the carbon and is readily available in large quantities from waste combustion gases, from fermentation processes, lime calcination, or even from carbon dioxide wells. The supply of carbon dioxide is ample, therefore, and the cost low. Water, of course, is necessary to supply the hydrogen and oxygen in the organic synthesis. Mineral nutrients and possibly some other micro-nutrients have to be added to the water to provide the optimum conditions for most rapid growth To conserve on costs, it was planned to reuse the spent culture medium by recycling the solution after the *Chlorella* had been separated from it, and after fresh nutrients and make-up water had been added. Fixed nitrogen can be supplied at the concentration necessary to control the synthesis for high protein or high fat, as desired.

Light, of course, is the source of energy which makes the process possible. Sunlight is always available and calculations showed that no consideration was warranted of any other source of light at present.

In addition to the raw materials required for the growth of *Chlorella,* certain environmental conditions must be maintained to achieve rapid growth. *Chlorella* will grow under wide variations in temperature but the optimum condition is from 20° to 25° C. The temperature should go no higher than this upper limit, but when no photosynthesis is taking place lower temperatures are no detriment. Agitation was also considered as a necessary condition because the density of *Chlorella* is higher than water and the cells will settle out if agitation of some type is not employed. The last necessary factor considered was that of maintaining sterile conditions. The importance of maintaining pure cultures was unknown, although evidence was available that contamination lowered the growth rate of *Chlorella.* The use of an open system was considered but without many years of study and research, it could not be determined satisfactorily whether conditions could be maintained that would permit exposure to all types of contamination and still permit rapid growth rates and a suitable final product.

Several important concepts were considered in order to visualize and develop an actual system or process for the most efficient production of *Chlorella.* To make a system successful it first must be technically feasible and, second, it should give a fair return per dollar of investment and operating cost. A system of large shallow tanks was visualized in which growth would be allowed to proceed for a period of time and the contents then harvested. A further development from this would be a series of tanks, each successively larger, to permit more efficient utilization of light, space, and equipment. Careful analysis of data on the growth of *Chlorella* clearly showed, however, that growth was most rapid during a short portion of the life of a batch culture. This indicated that for maximum production, the culture should be grown under conditions optimum for its most rapid growth and highest yield. Therefore, a continuous system was visualized in which conditions were maintained constant at the point for maximum production. Without experimental data, however, it was virtually impossible to accurately estimate costs of producing *Chlorella* and it was clear that no estimate could be made of the value of large-scale cultures without considerable reseach. A survey of the literature had shown that Dr. Jack Myers (Myers and Clark, 1944) had developed a laboratory apparatus that would maintain constant conditions and that studies could easily be made with this equipment to determine optimum conditions for growth. In addition to the importance of this technique for basic scientific studies, Dr. Myers has made a significant contribution in showing the technical feasibility of a continuous system. By holding conditions of culture constant and also by studying the effects of varying conditions on the growth of *Chlorella,* he has thrown new light on the mechanism of photosynthesis. His work provided strong evidence that a large-scale continuous system maintaining constant conditions was technically feasible.

The importance of maintaining a continuous system is easily seen. Sunlight, in order to be used most efficiently, must be incident upon a culture in which optimum conditions for photosynthisis are maintained. These conditions exist in a "batch culture" (one grown over many days, starting with a small inoculum and being harvested at a high population density), for only a limited period of time. In agriculture there is an extremely inefficient utilization of sunlight. The growing season is short and sunlight during the remainder of the year is wasted. Young plants intercept but a very small portion of light falling on the land. It is only during a short period before harvesting that a substantial portion of light is actually incident upon a surface capable of photosynthesis.

With algae, it is not difficult to visualize a system where constant conditions can be maintained in a continuous system permitting the most efficient use of land, sunlight, and equipment. By continuously harvesting at the rate at which the algae increase, and supplying the necessary raw materials, the optimum conditions may be maintained and the maximum possible production realized.

DEVELOPMENT OF CHLORELLA CULTURE PROCESS

Experimental

To become familiar with the problems of growing algae, to obtain a simple continuous system, and to maintain accurate control of all variables, a 4-inch diameter pyrex glass column, six feet in height, was constructed. This column (Figure 1) is illuminated by three 100-Watt fluorescent lights, placed about ¾ of an inch from the column wall, and spaced 90° apart around the inside of a sheet-metal reflector. A cooling tube is sealed into the top part of the unit and extends almost to the bottom. Temperature is maintained by a temperature controller which regulates the flow of cooling water. Aeration is provided by means of an inlet tube which enters at the bottom of the column. Two other tubes are sealed into the column near the bottom; one for the introduction of new medium and the other for sampling. Volume is maintained constant by an overflow at a level of 10 liters. The population density of *Chlorella* in the culture is held constant by means of a photoelectric cell with amplifier which activates a solenoid valve allowing the entry of new medium. This medium is supplied from glass bottles as indicated in Figure 1. A continuous supply of sterile medium is available from the supply bottles. By this technique, a closed sterile system is

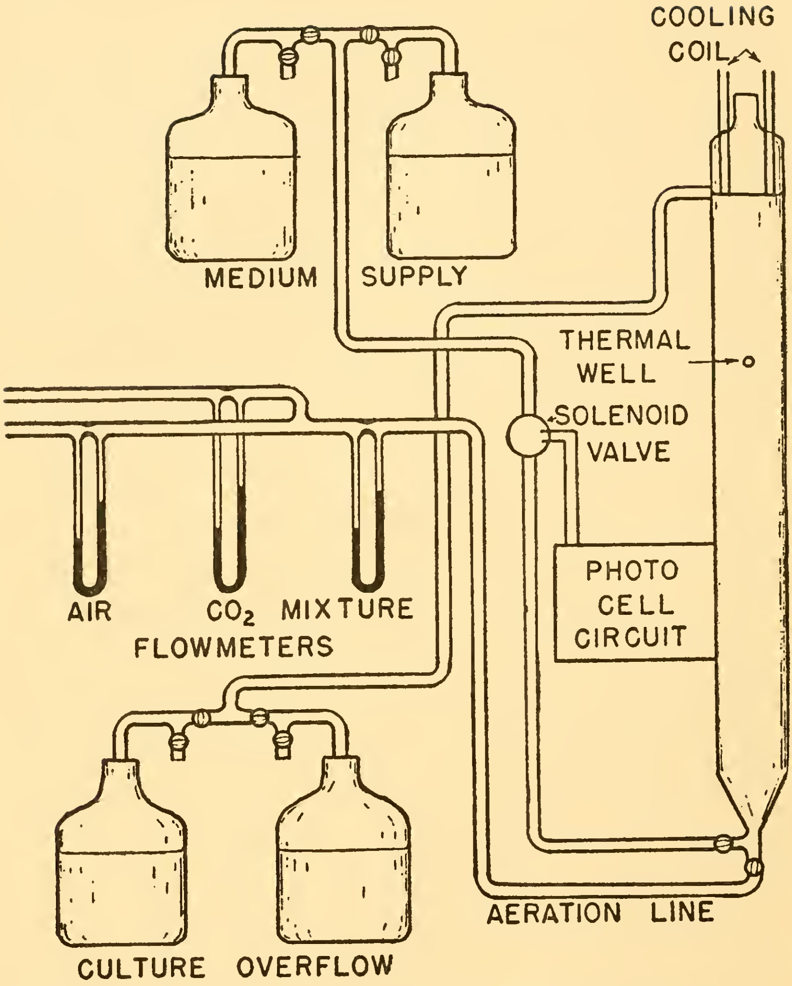

Fig. 1—Artificial light continuous culture apparatus in 4 in. column.

assured. The aeration mixture is controlled by pressure reducers and needle valves on a compressed air line and cylinders of compressed carbon dioxide. Each gas is metered through manometer flow-meters. Cotton gas filters are used, all connections are sterile, and the usual aseptic techniques are employed. A photograph of the apparatus is shown in Figure 2.

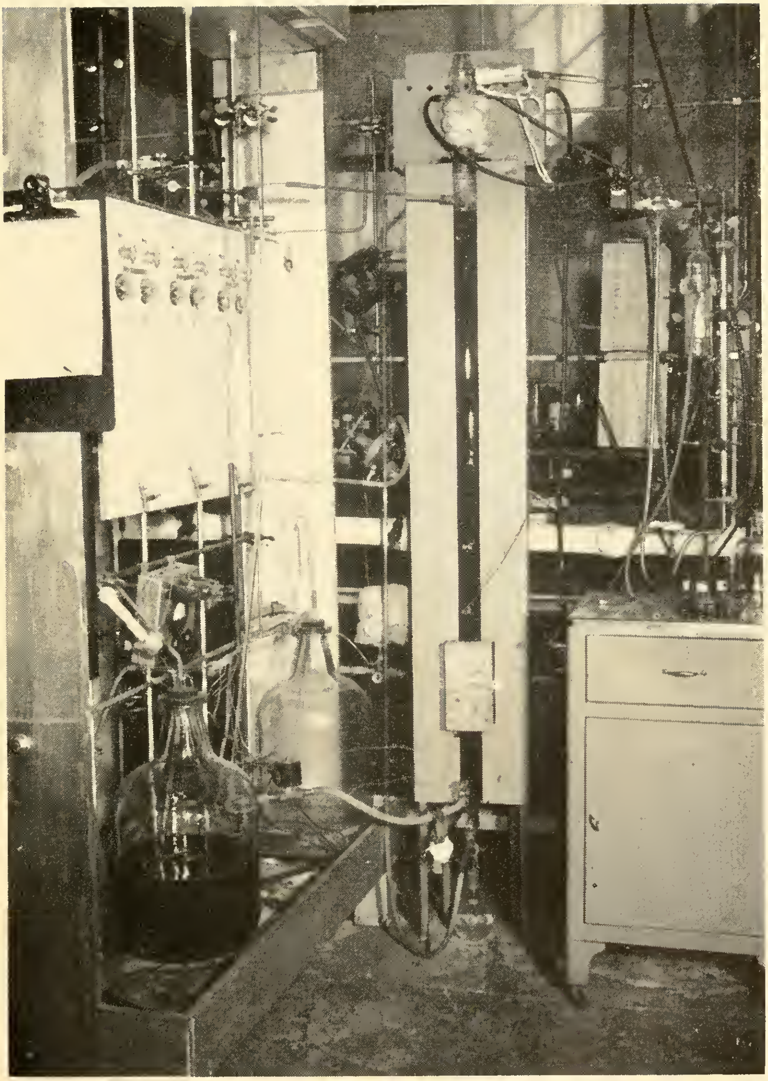

FIG. 2—Photograph of the apparatus illustrated diagrammatically in Fig. 1.

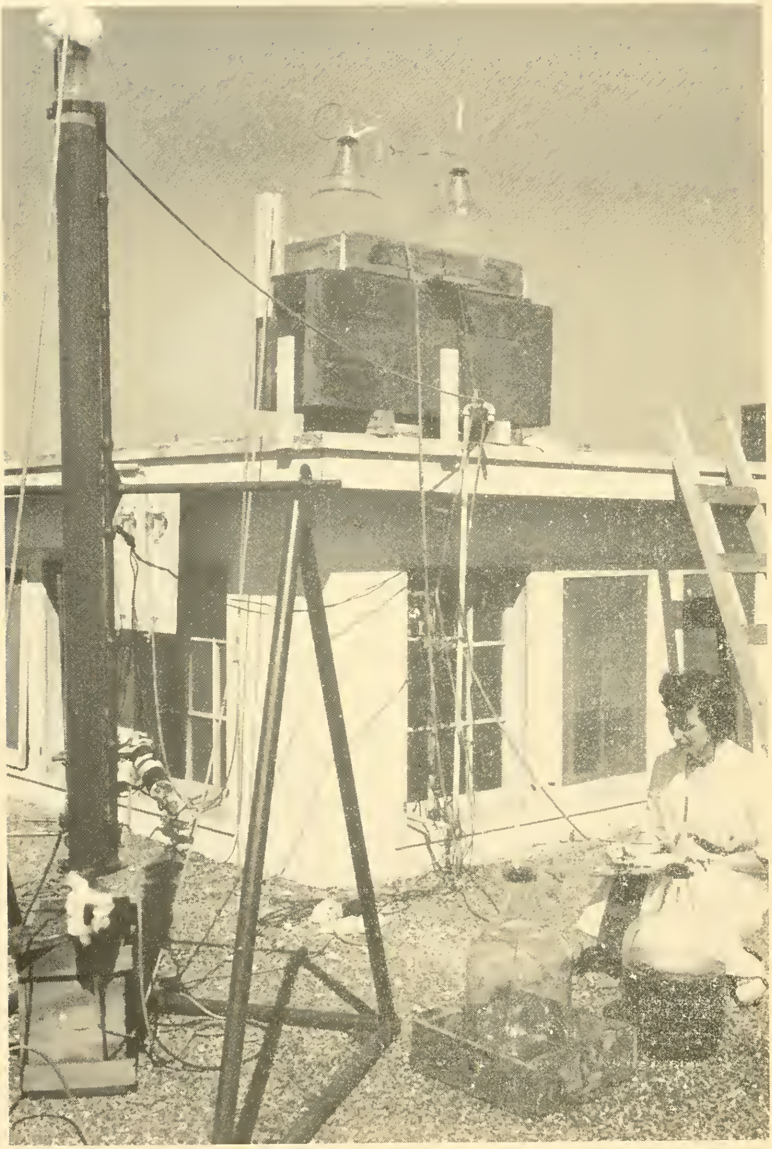

Fig. 4—Photograph of the apparatus including a 4 in. column.

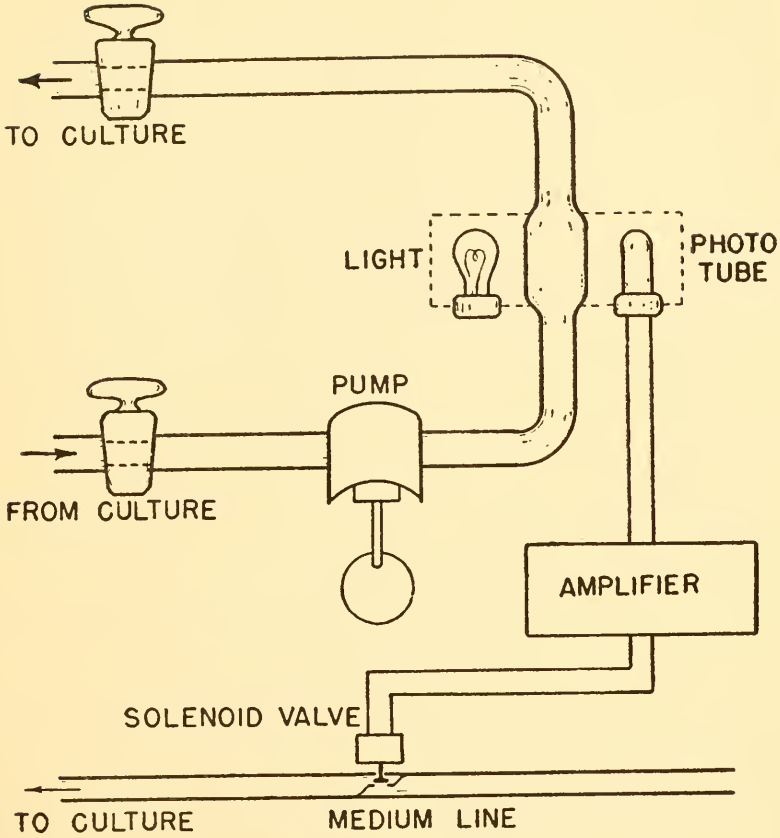

FIG. 3—Population density control, continuous culture process.

Experiments have been made with the apparatus since July, 1949. Variations in water and micro-nutrients, mineral nutrients, temperature, composition of aeration gas, population density of culture, and other variables have been studied.

To obtain information on growth in sunlight, a column 4-inches in diameter was constructed for operation out-of-doors. This column is similar in construction and operation to the artificial light, continuous column. It is mounted on a pivot so that the column may be operated normal to sunlight or in a vertical position, or at any angle desired. A separate by-pass, fitted with a pump, recycles medium through a cell enclosed in a black cylinder containing a constant-intensity light source and a photoelectric cell (See Figure 3). Constant dilution is obtained in the same manner as in the inside column. A photograph of the column in operation is shown in Figure 4.

Experiments have been made with this apparatus since September, 1949. Variables affecting rates of growth and production have been studied. Light in this case is not, of course, a constant condition and all data therefore must be interpreted on a basis of varying light conditions.

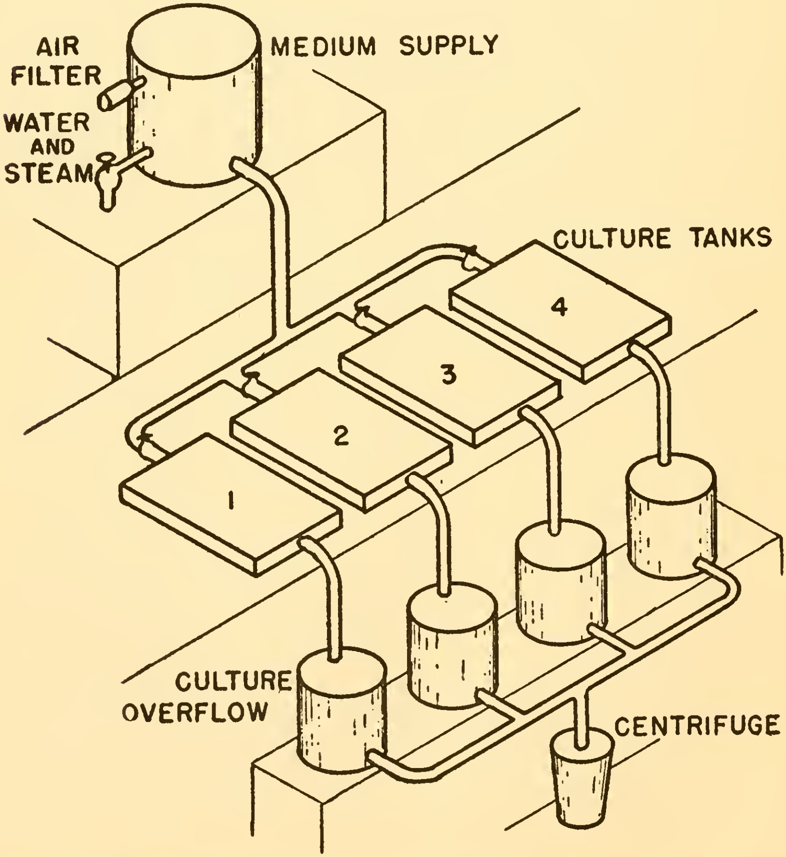

Fig. 5—*Chlorella* culture pilot plant, continuous culture process.

Pilot Plant

Results from data obtained in the above experiments indicated the optimum conditions necessary for maximum yield. These results were evaluated in terms of the best system for maximum production and minimum cost. A small pilot plant was then designed and constructed. The pilot plant is shown diagrammatically in Figure 5. It consists of four horizontal culture tanks four feet wide by ten feet long. The depth of the tanks is six inches. Each tank is maintained at constant conditions in the same manner as our other equipment. Barrels coated with non-toxic paint are used for medium supply and overflow storage. Tap water is constantly circulated through cooling coils welded to the underside of the tank. Temperature of the culture is maintained constant by a thermoswitch controlling a solenoid valve allowing steam to enter the cooling coils. A supply of carbon dioxide can be added to the medium or aerated into the tanks by means of six jet orifices in each tank. Constant population density is maintained by a photoelectric cell circuit in conjunction with a bypass in the system through which culture is constantly pumped. As new medium is allowed to enter, the overflow passes into the storage barrels thus maintaining a constant depth or volume of culture in the tanks. The overflow barrels are directly piped to a Sharples centrifuge for separation of the cells.

This pilot plant should serve two purposes: (1) to obtain substantial daily production of *Chlorella;* (2) to determine optimum conditions of growth in the type of system visualized for large-scale production.

Experiments are proposed employing the same conditions in each tank, except that the depth of the culture is varied. Therefore, the important variables of depth versus population densities during the same conditions of sunlight can be studied. The four tanks would be operated at culture depths of 1½, 3, 4, and 5 inches. By maintaining the same population densities in each tank, data of growth rates and yields versus depth of culture can be obtained for the same sunlight conditions.

Another phase of this research program has had the objective of determining the value of *Chlorella* as a source of foods or of chemicals. Work to date has been limited. Preliminary nutrition studies with rats have been made in which diets having all protein supplied by *Chlorella* were used. Feeding experiments with a normal complete diet supplemented with *Chlorella* also have been made. Some chemical biological analyses have been made both by our own laboratories and by other organizations.

Results

Only a brief and general report of our results is possible here. Only the important results as they pertain to the objective of producing the maximum quantity of *Chlorella* most efficiently on a large scale will be discussed.

Growth rate refers to the amount of growth in a culture per unit of time. In a batch culture growth rate is measured in terms of increase in cell count per unit volume per unit time. The growth rate in such a batch culture is not constant due to changing culture conditions and, therefore, is difficult to determine accurately. In a continuous system where population density and other variables are held constant, growth rate, expressed in liters of overflow of culture per liter per day, is measured by the amount of dilution (or the amount of overflow of culture obtained due to the dilution) necessary to maintain constant population density for a constant volume of culture. In a continuous system which maintains constant culture conditions, the growth rate is directly proportional to the rate of reproduction.

Growth rate is defined as:

$$K_D = \frac{V_O}{V_C} \frac{1}{\Delta t_D}$$

The yield (grams per liter per day) is the product of the growth rate (liters per liter per day) and the population density (grams per liter), and it is expressed as grams of material grown in a given period of time per liter of culture. It is also a direct expression of production in the continuous process. Yield is defined as:

$$Y_D = \frac{V_O}{V_C} \frac{D_C}{\Delta t_D} \quad \text{or} \quad Y_D = K_D D_C$$

The symbols as used are defined as follows:

K_D — growth rate (for 1 day)
V_O — volume of overflow in liters
V_C — volume of culture in liters
Δt_D — time in days (24 hours)
Y_D — yield (grams dry weight per liter per day)
D_C — population density (grams dry weight per liter)

Growth rate of high-protein *Chlorella* when maintained under constant culture conditions is a function of the population density and light intensity. Under the constant light conditions of the 4-inch artificial light column, we have made many experiments at different population densities. The results of one such experiment are shown in Figure 6 where volume of overflow is plotted versus time. The volume of overflow is constant with time and the slope of the line is the growth rate on an hourly basis. In several experiments particularly at high population densities a considerable period of time was required to reach equilibrium conditions and to obtain a constant increase in overflow volume per unit time.

Figure 7 shows the growth rate versus population density for several experiments conducted in the artificial light 4-inch column (as above) with optimum culture conditions. Yield, the product of growth rate and population density in grams dry weight per liter, is plotted versus population density in Figure 8. This curve shows a maximum yield at a density of 0.36 grams dry weight per liter. The maximum production obtained from such a system is 0.48 grams dry weight per liter each day.

Similar information has been obtained with the sunlight 4-inch column. The results of one experiment are shown in Figure 9 where overflow volume is plotted versus time and date. The daily weather conditions and overflow volume are indicated. This experiment was carried out at a population density of 0.27 grams per liter with the column perpendicular to the earth. The daily average yield for this experiment was 0.279 grams per liter and

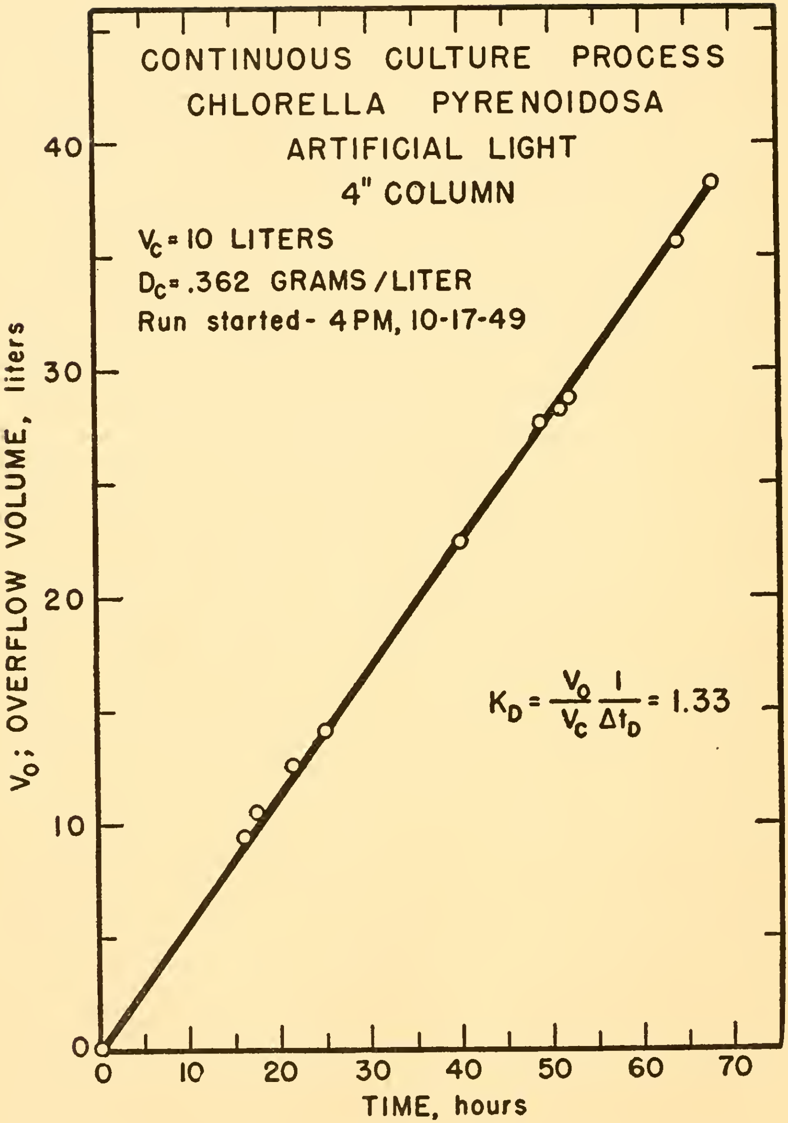

FIG. 6—Continuous culture process using *Chlorella pyrenoidosa* under artificial light; 4-inch column. Overflow volume vs. time.

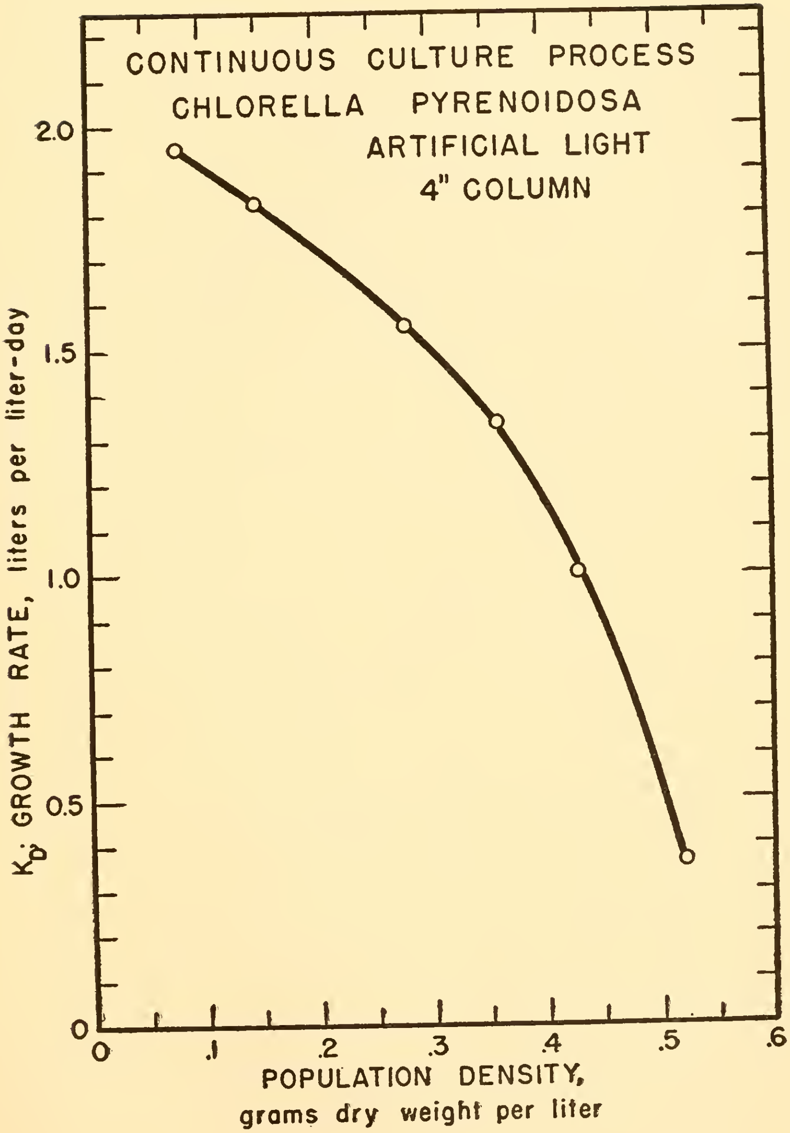

FIG. 7—Growth rate vs. population density, artificial light, 4-inch column.

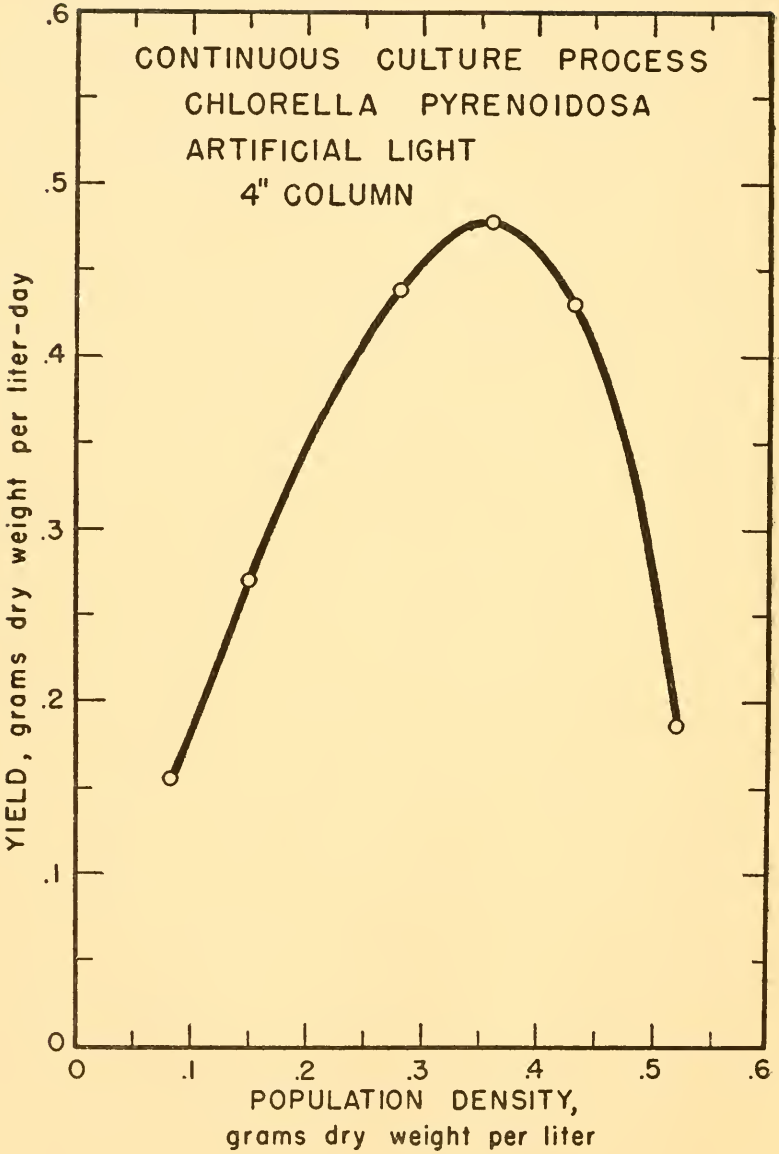

FIG. 8—Continuous culture process using *Chlorella pyrenoidosa* under artificial light; 4 in. colum. Yield plotted against population density.

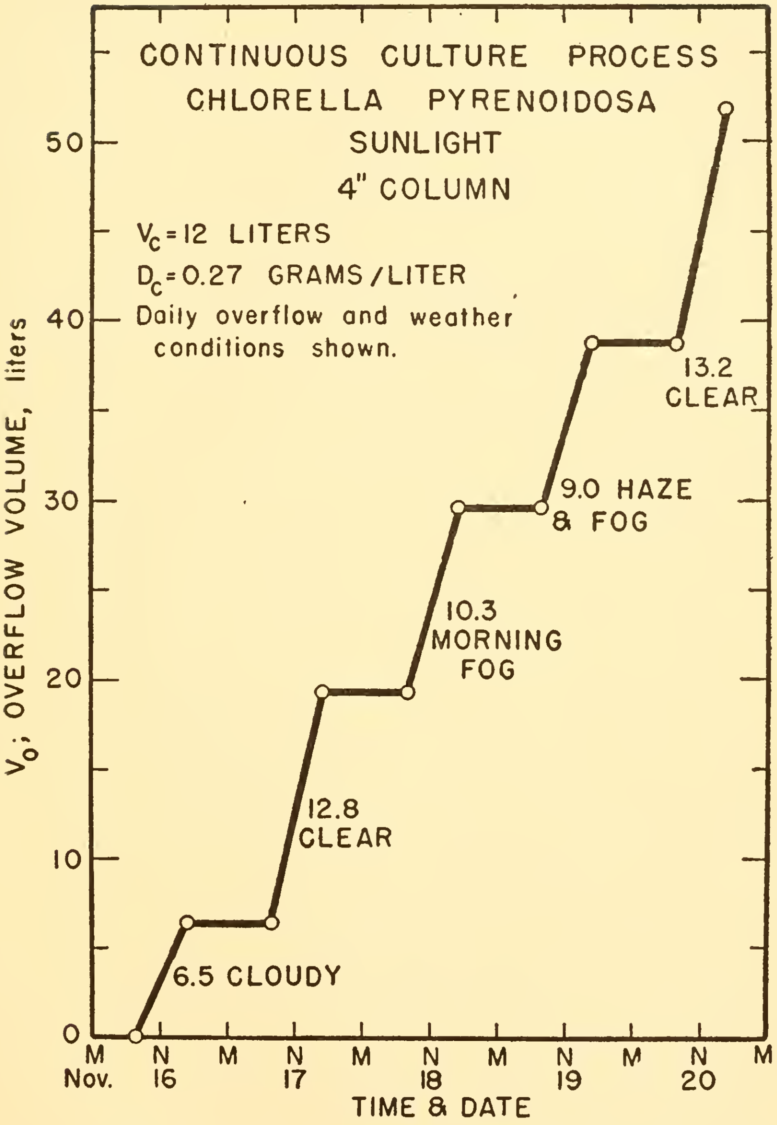

FIG. 9—Continuous culture process using *Chlorella pyrenoidosa* under sunlight; 4 in. column. Overflow volume plotted against time.

the yield for clear days was 0.351 grams per liter. The total culture intercepts two square feet of sunlight on a vertical plane and 89% of the incident sunlight is transmitted into the column. Actual growth rates for this equipment are considerably higher than the artificial light column due to the higher intensity of sunlight during the day.

Some data have also been obtained for different values of the following variables:

Carbon dioxide - air mixtures for aeration; aeration rates and turbulence; temperature; light; mineral nutrients and pH; water and micro-nutrients; contaminants.

The optimum nutrient and culture conditions for large-scale growth as determined by us do not vary seriously from the present common practices of *Chlorella* culture. Five per cent carbon dioxide in air appears to be the best aeration mixture. There was no detectable change in rate of growth when the carbon dioxide was varied ± 1% in our apparatus. An optimum temperature of 25° C. confirms some results given in the literature. An aeration rate of 3 cubic feet per hour or more in the 4-inch column is optimum, but this, as will be discussed later, is apparently dependent upon the turbulence necessary rather than the carbon dioxide required. The mineral nutrients are optimum over a wide range and agreement is found with the work of Spoehr and Milner (1949) and many others. By using water from different sources we obtained different rates of growth even after supplementing with Arnon's A-4 and B-7 micro-nutrient solutions. We have not yet been able to detect what other micro-nutrient, or what quantities of it, influenced this variation in the growth of *Chlorella*. In one case, it is quite definite that it was the presence of one micro-nutrient in too high a concentration, rather than a lack of it, that had an adverse effect on production. The water which permitted the most rapid growth was always used for study of other factors.

Our cultures have become contaminated on several occasions and in some cases this may have caused lower growth rates than normal. A continuous culture, however, has now been maintained for over two months without contamination. There is no reason to believe that this period could not be extended indefinitely with the proper precautions and techniques.

A considerable volume of data has been obtained concerning the variables in the culture of *Chlorella*. All this information has been or will be used in the determination of the most efficient and economical process and plant for the large-scale culture of *Chlorella*.

Interpretation of Results

In developing a process for the large-scale culture of *Chlorella,* it is necessary first to consider the limitations as determined by nature. Because solar radiation provides the energy, we are limited to the use of sunlight as received on the earth at the particular geographical location in question. This, of course, varies with the time of day and year as well as with atmospheric conditions. We are also limited by the mechanism of photosynthesis and the physiology of the organism to be considered. Within these limitations or parameters, a process must be developed which will most efficiently produce organic matter using the sun's energy. In other words, how can we most efficiently use sunlight to synthesize organic matter?

From a study of solar radiation and a study of the mechanism of photosynthesis as it occurs in the organism of *Chlorella,* we have developed a tentative process to attain this objective. I do not have the space here to discuss the data and results that have contributed to this design, but I will mention the more important factors.

Light

Light from the sun supplies the energy for the conversion of carbon, oxygen, hydrogen, and nitrogen to *Chlorella*. The intensity of sunlight varies from time to time over a wide range so that there is no one light condition to be studied in reference to maximum conversion. It has been demonstrated (Myers and Burr, 1940) that *Chlorella* exposed to light of high intensity will grow rapidly at first, then at a diminishing rate until injury takes place and death of the cell occurs. For a given culture the completeness of this cycle is a function of the length of exposure and the intensity of the light. It also has been shown (Myers, 1946; Myers and Burr, 1940) that at a certain critical intensity of light and above, a cell is light-saturated and has reached its maximum rate of photosynthesis. At a lower critical intensity, the maximum growth rate is reached (Myers, 1946; 1946a). Therefore, we can expect to gain no further benefit from increased light intensity, with the exception of having more volume growing at maximum rate due to greater penetration of light through the culture. We have demonstrated that at various wave lengths and intensities of light a *Chlorella* culture closely follows Beer-Lambert's Law on the absorption of light. This will show that at high light intensities *Chlorella* absorbs considerably more light than can be used, due to the light-saturation point of each cell. In a practical system, then, under high light intensities, a considerable portion of the light will be wasted. We can visualize no practical system to overcome this inherent difficulty. More data still must be obtained on the light-saturation point of *Chlorella* under various conditions, but it is now clear that as much as 85% of the light is wasted under certain conditions of population density and culture, even though the culture is deep enough to permit light extinction. This seriously limits the possible amount of conversion of light in the synthesis of organic matter. In spite of this we have succeeded in converting approximately 2½% of the incident solar radiation in the production of organic matter. Some improvement may be expected by maintaining a greater depth of culture in the new pilot plant, inasmuch as the culture in the column is not absorbing all the light at times of maximum intensity.

POPULATION DENSITY

For maximum production in sunlight under the best culture conditions now known, population density should be maintained at 0.36 grams dry weight per liter. For all practical purposes, this can vary by ± 10% without an important reduction in yield. If a system allows a wider variation of population density, an appreciable reduction in yield is incurred. If conditions can be found that will permit the same production at high densities, certain obvious savings in equipment and processing will result. It has been one of our objectives to determine conditions that will permit the same production under more dense culture conditions, but to date, we have not been successful.

Turbulence

A necessary condition for optimum growth is turbulence or agitation. This serves three primary purposes: 1) to keep cells in suspension and, therefore, at uniform density; 2) to maintain equilibrium conditions between the necessary nutrients and each cell; and 3) to remove cells under high light intensity conditions to lower light intensities to prevent their injury and thus maintain them at maximum growth rates. This can be obtained by mechanical or gaseous agitation or by turbulent flow conditions of the culture medium.

Other Conditions

Temperature must be maintained at 20° to 25° C. plus or minus a few degrees for maximum production. During periods at night with no illumination we have not observed adverse effects after allowing the temperature to fall as low as 7° C.

A closed and sterile system is necessary to maintain an uncontaminated culture. Because there are indications that certain molds and bacteria will cause a considerable decrease in growth rates, such a sterile system is certainly necessary in a large-scale process at present. Considerable work and years of experimentation might provide a system that would operate without aseptic techniques. Even when such a system is devised, however, contaminants not previously encountered might destroy a culture and bring about costly expenditures. Therefore, not only must a closed sterile system be used, but also one in which contamination can be localized and the system easily cleaned and resterilized without sacrificing a large amount of the culture or incurring high labor costs.

The supply of nutrients must be provided at the optimum levels within the range of concentration of each nutrient that permits best growth. Carbon dioxide would be supplied to the medium at the proper concentration. Fixed nitrogen as ammonium ion or nitrate ion would supply the nitrogen. Ammonium is the most promising, primarily for economic reasons. Other nutrients required are minor and present no problems and so will not be discussed here.

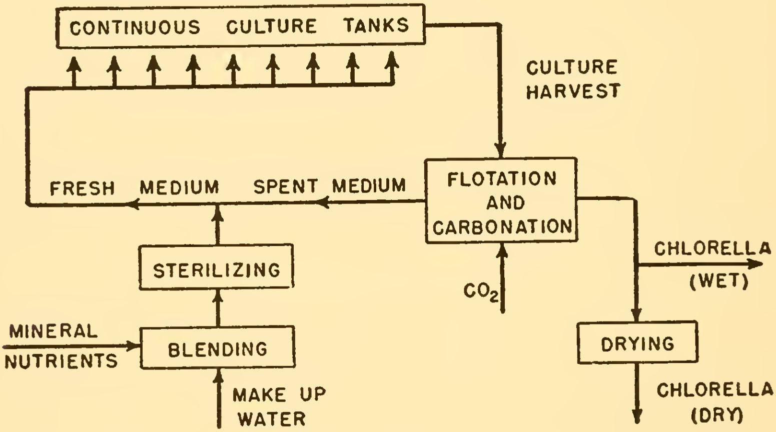

FIG. 10—Flow diagram of the continuous culture process.

LARGE-SCALE *Chlorella* CULTURE PROCESS

The major conditions have been considered and must now be incorporated into a simple, cheap, efficient system. This requires not only inexpensive construction, ease of control and operation, but also maximum efficiency. A continuous process is, in our opinion, the only way to obtain such a system. Conditions must always be maintained at the optimum point. Each cell must receive the required nutrients, proper population density must be maintained, and the temperature must be correct. To obtain a continuous system is not difficult but to design the most efficient system requires the consideration of a large number of complex variables. We have studied and experimented with these variables at some length. The tentative plant design I should like to describe is one we believe to be the most efficient and most economical we have constructed thus far. Certainly, the basic principles will apply to any design. The details of the engineering are not important and will not be discussed.

In Figure 10 is shown a simple block flow diagram illustrating the major ideas involved. There are two major parts of the plant: 1) the culture farm; 2) the processing plant. Growth takes place in long, flat, horizontal, glass-covered tanks. Conditions for growth are maintained at optimum by introducing fresh medium at frequent intervals along the tank. Because growth is constant in all portions of the tank it is necessary to provide an equal flow of fresh media into each inlet of the tank, thus maintaining constant population density. When each interval is equally diluted and culture is removed only at the end of the tank, the flow rate of the culture is necessarily low near the head and rapid near the end. To obtain proper agitation, the culture is recycled in enough quantity to obtain a condition of turbulent rather than streamlined flow in the tanks. The necessary nutrients, including carbon dioxide as carbonate and bicarbonate, are introduced with the fresh medium. An atmosphere of carbon dioxide and air is maintained over the culture. The harvested culture flows to the processing plant where the cells are separated from the medium by flotation. Waste combustion gases from the sterilization and dehydration processes supply enough carbon dioxide for growth requirements. Some of these gases are absorbed in solution during the same flotation process for removal of cells. Make-up water to which the necessary nutrients have been added, will be sterilized and incorporated with the spent medium for return to the culture farm.

One particular design for the tanks is shown in Figure 11. The tanks operate by gravity flow. Each tank extends to the end of the farm, turns, and returns to its origin at a slightly lower level. Here the recycled portion is pumped up to the head of the tank. A pipe carrying new medium extends most of the length of the tank and introduces the new medium at equal intervals in the tanks. Thus, we have a simple, continuous system. It provides for

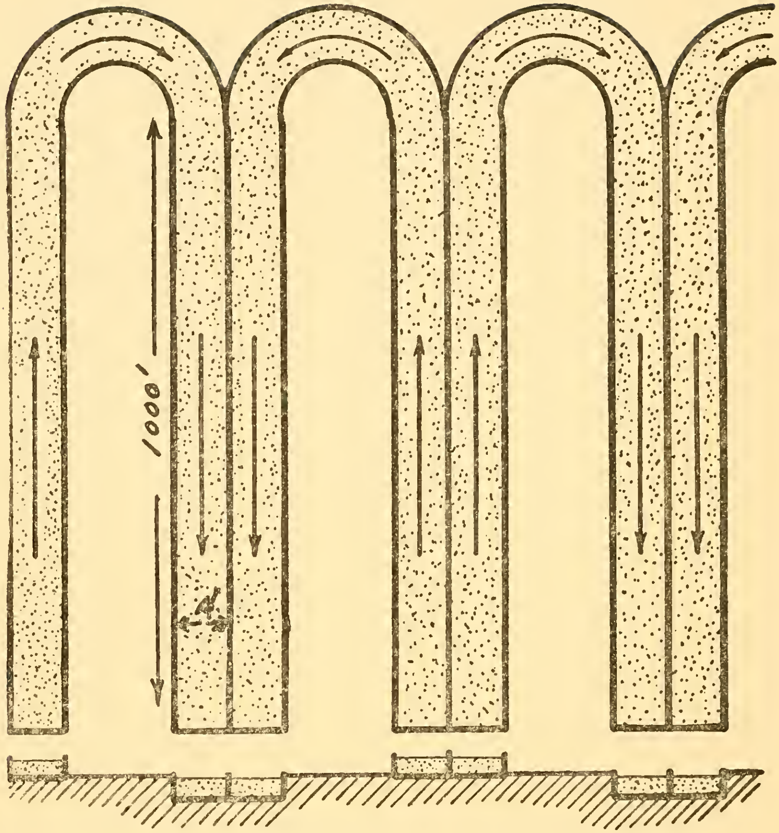

Fig. 11—Horizontal tank farm in the continuous culture process.

optimum conditions at all points because the flow of fresh medium into the tanks depends on the growth and, therefore, on the intensity of sunlight. Control is maintained by a simple photoelectric cell circuit.

Future Work

A very considerable amount of work, primarily of a basic nature, still remains to be done. Studies on the influence of light are particularly important, including examinations of growth rates versus light intensities at high light intensities, and the determination of the growth mechanism when the cell is light-saturated. Additional information on obtaining better growth rates at high population densities and on the effects of contamination are certainly needed. A clearer picture of the inhibition of growth by metabolic products would be an important contribution. There is no reason to believe that a better organism than *Chlorella* might not be found.

The general field is certainly an exciting and promising one; one that in the next few years will show important advances. Slowly it is being realized that photosynthesis has a potential of great import when harnessed and properly utilized. The amount of money spent on research in this field has been small but already the results show great possibilities.

SUMMARY

As the world becomes more and more dependent on energy received from the Sun to synthesize its life-giving products, ways must be devised to obtain greater efficiency in utilizing this energy. Agriculture, even under the most favorable conditions, converts but 3/10ths of 1% of a year's solar radiation to organic matter, one half of which is essentially waste material (Daniels 1949). One promising method for producing large quantities of protein, lipid, and carbohydrate has been presented in this paper. On a small scale, as we have seen, 5% of the incident sunlight, or over 2½% of the total solar radiation, has been converted to organic matter. Excellent possibilities exist for improvements from both short term and long range studies.

The system we have described may not be the best nor the organism used the most efficient. Only future studies can determine these matters. The important fact is that photosynthesis has been used and controlled in a simple process. It can be harnessed by Man to produce needed products. We can look forward to new and better ways of putting photosynthesis—and the boundless source of energy provided by the Sun—to work for us.

CULTURING OF MARINE ALGAE IN RELATION TO PROBLEMS IN MORPHOLOGY[4]

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Up to the present, the green, brown and red marine algae (the only groups taken into account in this article) have been cultured primarily with the purpose of gaining information concerning their developmental morphology and life history. The methods have been crude as judged by the standards of the physiologist, who has to have pure, bacteria-free cultures. But the achievements none the less have been significant, not only with respect to the initial objectives but in the profitable use that has been made of the knowledge obtained for the interpretation of phylogenetic relationships within the groups.

Culturing Methods and Solutions

Ordinarily, cultures are started from reproductive cells—zoospores, aplanospores, gametes, or zygotes. Fertile plants are placed in dishes of filtered sea water and the liberated reproductive cells are allowed to become attached to slides placed along the sides or at the bottom of the containers. In other instances drops of sea water containing reproductive cells are placed on slides or cover glasses and the cells allowed to become attached. It is always necessary, of course, to determine the nature of the cells to be cultured. Are they asexual spores, gametes, or zygotes? Consequently, it frequently is desirable to start the cultures in hanging drops, which facilitate observation of the behavior of the cells and the early stages in their germination.

The slides or cover glasses with the attached cells are transferred to dishes containing the nutrient solution and placed in a cool room with ample light. Direct sunlight should be avoided and the culture solution should be renewed periodically. In certain instances it is desirable to aerate the cultures.

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[4] This paper was prepared during the tenure of a Guggenheim Fellowship.

The nutrient medium which has yielded the most satisfactory results is Erdschreiber solution. It was originally prepared by Føyn (1934, pp. 6-7), although the formula was first published by Hämmerling (1931, p. 635; see also 1934, p. 4). This medium is much the same as the one used previously by Schreiber (1927), but differs in the substitution of soil extract for distilled water (hence "Erdschreiber"); and the one of Schreiber was in turn the medium of Pringsheim (1921) which was modified for use with marine organisms. Erdschreiber solution consists of: sea water, 1 liter; soil extract, 50 cc.; $NaNO_3$, 0.1 gr.; Na_2HPO_4, 0.02 gr. (For the method of preparation of soil extract see Hämmerling, 1931 and Føyn, 1934.) Kylin (1933) and others added to Schreiber's medium 3-5 drops of a 1 per cent solution of ferric citrate.

Although it has been established by Pringsheim (1936a) that the growth-promoting substances are organic compounds, their exact nature has not yet been determined (Pringsheim, 1949, p. 42). Kylin (1942) believed that ascorbic acid may be concerned here, at least in part, and he showed (Kylin, 1946a) that in addition to ascorbic acid, aneurine and heteroauxin also strongly stimulate growth in *Ulva.*

Schreiber (1935), Suneson (1942, 1943) and Kylin (1943) found that the addition of algal extract to the solution of Schreiber enhanced growth; and de Valéra (1940) and Kylin (1941) established that in-shore surface water, especially from the *Fucus-Ascophyllum* zone, was much richer in growth-promoting substances than off-shore water or water from a depth of 35-40 meters.

Artificial sea water has been used by a number of investigators in the cuturing of algae, sometimes with a good deal of success. Dostál (1929), for example, found that *Caulerpa* not only grew in it but the plants became fertile. Formulae for the preparation of artificial sea water and references to the extensive literature on the composition of sea water may be obtained from the works of Schiller (1928), Sverdrup, Johnson and Fleming (1942) and Levring (1946).

Chlorophyta[5]

Since 1925, when Miss Williams showed that *Codium* was a diploid alga, our concepts of the life cycles of green algae, especially the marine forms, and of the associated nuclear phenomena have undergone a profound change. It is now known that the Siphonales and Dasycladales are diploid algae, or at least those for which the cytology has been studied, and that it is the diploid soma which in them, as in the Fucales and in animals, functions as the gamete-producing generation. In certain other orders, such as the Cladophorales *(Cladophora, Chaetomorpha),* Ulvales *(Ulva, Enteromorpha),* Ulotrichales *(Draparnaldiopsis, Fritschiella;* Singh, 1945 and 1947, respectively) and Siphonocladales *(Anadyomene, Microdictyon;* Iyengar and Ramanathan, 1940 and 1941, respectively) there are forms the life cycle of which includes an alternation of isomorphic generations; and Juller (1937) has shown that *Stigeoclonium subspinosum* Kütz. (Ulotrichales) possesses an alternation of heteromorphic generations, with the diploid, asexual generation smaller than the haploid one.

Although the green algae have not been cultured as extensively as would be expected, there are many advantages in this method of approach in the study of problems in this group, as may be gathered from the following brief review of some of the work that has been done.

Following the discovery of an alternation of isomorphic generations in the life cycle of members of the orders Cladophorales and Ulvales by Hartmann (1929) and Føyn (1929, 1934, 1934a), several genera and a variety of species of these two orders have been found to possess a similar cycle. However, it has also been shown, that there are forms which deviate from this seemingly general rule. Through a long series of papers Bliding (1948, 1948a, and earlier) has tremendously advanced our knowledge of the life cycle and taxonomy of species of the genus *Entero-*

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[5] See Papenfuss (1946) on nomenclature for the phyla of algae.

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morpha. He has found that some of the species, and races of others, lack a sexual generation and reproduce by asexual spores only. In some instances, these are biflagellate and resemble gametes, as in *E. biflagellata* Blid. (Bliding, 1944), and in others, such as *E. procera* Ahln. (Bliding, 1933), they are quadriflagellate. Bliding (1938) has also shown that in its early development *E. minima* Näg. differs from other species in the possession of an extensive prostrate system; and on the basis of this character Kylin (1947) later removed this species to a new genus, *Blidingia.*

Although many of the species of *Enteromorpha,* particularly as represented in herbarium specimens, probably will continue to defy precise identification, the cultural work of Bliding clearly shows that from the point of view of life cycle and ontogeny, marked differences exist between a number of the species.

Monostroma is another genus of the Ulvales which has been the subject of morphological study in recent times, with extremely interesting results. As early as 1878, this genus was studied in culture by Reinke (1878a) who found that the plants obtained from the sea were sexual in nature. The zygotes became resting zygospores, the content of which in certain instances was divided into cells after a period of 7 to 8 weeks. Reinke thought that this was an early stage in the development of a *Monostroma* thallus.

Kunieda (1934) was the first to show that after a rest period of about five months, the thick-walled zygospores each formed about 32 quadriflagellate zoospores. These observations have been confirmed by Yamada and Saito (1938) and by Moewus (1938), the latter having shown also by cultural methods that half the zoospores gave rise to female and half to male plants.

The investigations of these authors proved conclusively that *Monostroma* lacked an alternation of generations. Thus in its life cycle this genus departs from *Ulva* and *Enteromorpha.* Kunieda suggested that a separate family, Monostromaceae, should be created for *Monostroma,* an opinion with which Suneson (1947) and Kylin (1947) concur. In fact, Kylin (l.c.) even suggested that the Monostromaceae be removed from the Uvales to the Ulotrichales, which in general are characterized by the type of life cycle shown by *Monostroma,* although in his recent work

(1949) on the green algae of the west coast of Sweden he returned the family to the Ulvales, without comment.

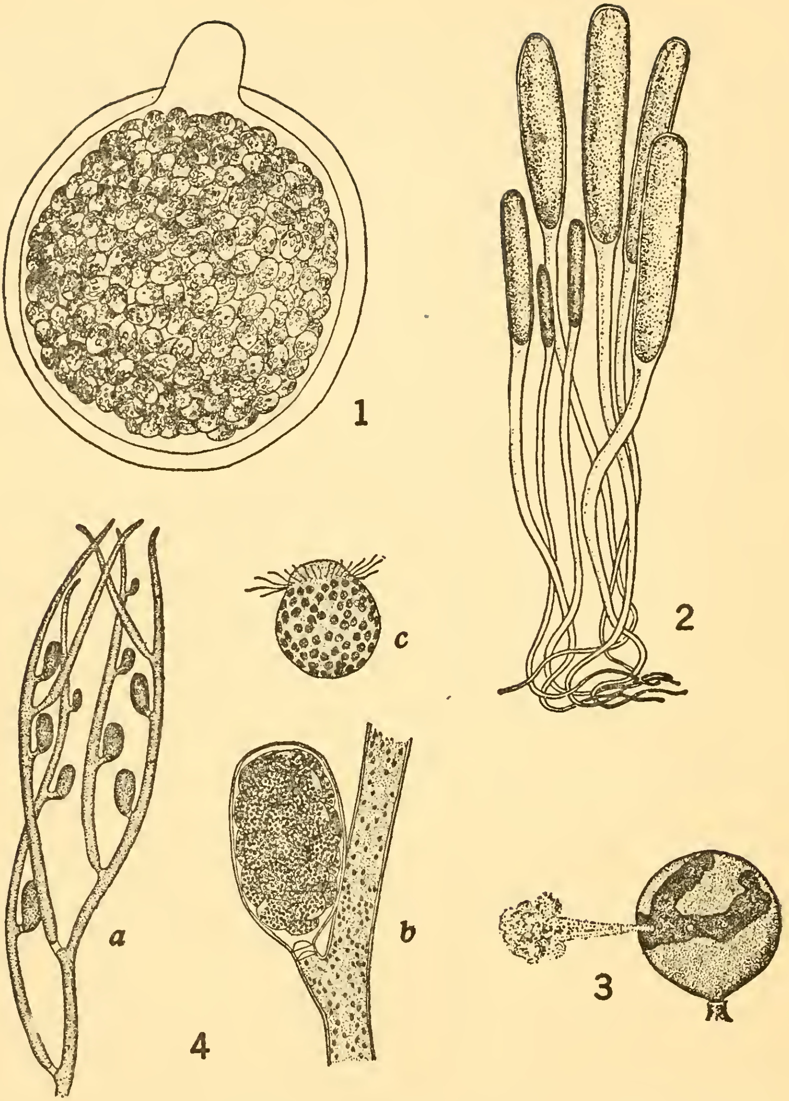
Yamada and Saito noted some interesting deviations in the reproductive phase of two Japanese species of *Monostroma.* The zygospore of *M. angicavum* Kjell. produces a comparatively large number of zoospores (fig. 1) and *M. pulchrum* Farl. lacks a sexual phase. The plants obtained in nature produce quadriflagellate zoospores, which form cysts. Following a resting period of about eight months and a half, each cyst gives rise to a large number of quadriflagellate zoospores which produce new plants. Quadriflagellate zoospores are thus formed at two stages in the life history of *M. pulchrum* Farl.

In 1940, Moewus reported that subjecting the mature zygospores of *M. Wittrockii* Born. to gametophyte extract caused the zoospores, formed later in the cysts, to behave as gametes and in some instances to produce two instead of the usual four flagella. On the other hand, a similar treatment of gametes with zygote extract did not cause them to lose their sexual power.

Jorde (1933), working on the west coast of Norway, has brought forth fairly conclusive evidence indicating that certain species of the unicellular *Codiolum* (fig. 2), a genus of the family Chlorococcaceae in the order Chlorococcales, represent the diploid asexual generation of species of the filamentous *Urospora,* a member of the family Cladophoraceae in the Cladophorales. These observations still require confirmation, but if they should prove to be correct, we would have here an alternation of hetermorphic generations, representing two kinds of green algae which for a long time have been regarded as phylogenetically very far apart.

In this connection attention should be brought also to the claim of Kornmann (1938) that *Halicystis* and *Derbesia* constitute phases in the life history of one and the same alga. These genera have been regarded as the type representatives of two distinct families, Halicystidaceae and Derbesiaceae, of the order Siphonales.

Previous to the work of Kornmann, Hollenberg (1935) had shown conclusively that the vesicular thallus of *Halicystis* (fig. 3)



FIGS. 1-4. FIG. 1—*Monostroma angicavum.*, zygospore which has produced zoospores. (× 650) From Yamada and Saito, 1938. FIG. 2—*Codiolum gregarium,* habit of plants. (× 30). From Newton, A handbook of the British seaweeds, 1931. FIG. 3—*Halicystis ovalis,* fertile plant liberating gametes. (× about 2.3). FIG. 4—*Derbesia marina,* a. portion of thallus; b. sporangium containing zoospores; c. zoospores. (a, × about 24; b, × about 245; c, × about 485). FIGS. 3, 4—from Smith, Cryptogamic botany, Vol. 1, 1938.

is gametophytic in nature. The plants are dioecious and form terminally biflagellate anisogametes. The zygotes form *Vaucheria*-like filaments which produce erect branches of limited growth and rhizomes which penetrate encrusting corallines, the customary hosts. Hollenberg (l.c.) believes that the rhizomes give rise to *Halicystis* plants on the surface of the host.

Starting with the stephanokont zoospores (fig. 4c) of the siphonous *Derbesia* (fig. 4a), Kornmann found that these cells gave rise to non-septate filaments which in the course of time produced vescicles which in every respect resembled plants of *Halicystis*. These vesicles became fertile in the same way as *Halicystis* does and formed zooids which resembled the gametes of this genus. Although no conjugations were observed, Kornmann believes that the zooids actually were gametes, because new plants developed only in those cultures which contained both large and small zooids. The "zygotes" produced *Vaucheria*-like filaments which were believed to be early stages in the development of *Derbesia* plants.

In view of the differing conclusions of Hollenberg in respect to the life history of *Halicystis,* it is evident that the work of Kornmann is in urgent need of repetition. In the opinion of the present author the great difference in the type of flagellation in the motile cells of *Halicystis* and *Derbesia* would suggest no close connection between these genera. In fact, *Derbesia* is almost as much of a misfit in the Siphonales as was *Vaucheria* before Eubank (1949) removed it to the Xanthophyceae, where it at one time had been placed.[6]

Prominent among the more outstanding work of recent times on marine Chlorophyta, is that of Hämmerling (1931, 1932, 1934, 1943, 1944, 1946, and other papers) and his associates (Schulze, 1939; Beth, 1943; Maschlanka, 1946) upon *Acetabularia* and other Dasycladales. A significant feature of this work is that it has not only extended immensely our knowledge of the life cycle and development of a very remarkable group of organisms, but it touches upon questions of fundamental importance to all biology.

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[6] In an article which appeared since this paper was written, Feldmann (Compt. Rend. Acad. Sci., Paris, 250 (3): 322-323. 1950) upholds the contention of Kornmann that *Derbesia* and *Halicystis* are phases in the life history of one and the same genus.

These investigators have succeeded in growing representatives of this order of tropical algae, especially species of *Acetabularia*, in culture for several generations. (For methods of culture see Hämmerling, 1934.) Thus they have had available at all times an abundance of material of known parentage and age, which has enabled them to study a variety of problems under well-controlled conditions. Of the many interesting facts that have been learned, the following merit special mention.

In contradiction to the long-standing belief that these plants are multinucleate, it has been found that they are uninucleate, with the possible exception of *Cymopolia barbata* (L.) Lamour. (cf. Hämmerling, 1944). The nucleus is large and lobed and is located in one of the arms of the basal rhizoid. The plants are diploid. When they become fertile, the primary nucleus divides mitotically and the so-formed secondary, smaller nuclei migrate up the axis and into the gametangia. Each of the operculate cysts that are formed in the gametangia contains at first a single nucleus. This nucleus next undergoes a series of mitotic divisions and later all the nuclei divide meiotically. The swarmers that are ultimately produced in the cysts are gametes.

A particularly convenient feature of *Acetabularia* is that the cysts, zygotes or sporelings will remain dormant or become so if kept in darkness. It was found that zygotes of *A. crenulata* Lamour. would still germinate after having been in darkness for eight and one-half months, that the cysts would liberate gametes after storage in darkness for as much as a year, and sporelings that were two and one-half months old remained alive in darkness for as long as eight and one-half months. Another very advantageous feature is that the mature cysts can be induced to liberate their gametes by a short treatment with distilled water. It is of great interest that in culture the plants fail to become calcified. The reason for this is still unknown but it immensely facilitated the cytological and experimental work.

The capacity of *Acetabularia* and *Dasycladus* to regenerate lost parts has been known for a long time. For this reason these algae provide particularly excellent material for experimental work; and they have been used to good advantage by Hämmerling and his associates. Hämmerling has found that if the terminal end of *Acetabularia* is removed from the basal, nucleus-containing portion, it will continue to live for 100 to 150 days; and if it had not yet formed a disc but was in an advanced stage of development, it would produce a complete disc at the distal end and at times one at the opposite end also. Neither of these discs would attain the maximal size characteristic of the species, however, and in the absence of nuclei no cysts are produced in the chambers of the discs.

It is of great importance to note that discs that are intermediate in the characters of two species are produced by grafts between parts of two species each of which contains a nucleus, or between parts of two species only one of which contains a nucleus, or between parts of two species neither of which contains a nucleus.

From these and other experiments Hämmerling concludes that the nucleus produces form-building substances or their precursors which function in the ontogenetic development of the plant. It is apparent, furthermore, that these substances are formed in advance of the time that their rôle in ontogeny becomes apparent.

Brief consideration is given below to a few of the innumerable problems in the morphology and taxonomy of the green algae that especially call for a study of plants grown in culture in addition to specimens obtained from the sea.

Some of the genera and many of the species representative of the orders Volvocales and Chlorococcales and the family Chaetophoraceae of the Ulotrichales are based upon very insecure foundations. Kylin (1935) and more recently Mrs. L. Moewus (1949) cultured a few of these algae and were thus able to determine the limits of the species in a much more satifactory way than heretofore had been possible. It would seem that many more of these algae could be similarly studied to great advantage. The excellent results of Bliding in his culture work on *Enteromorpha* suggests that the equally troublesome genus *Cladophora* should be studied along the same lines.

A few accounts in the literature (see Hurd, 1916, pp. 117, 118) indicate that the large swarmers of *Codium* may germinate directly at times. The question arises, are these cells female gametes which develop parthenogenetically or are they zoospores? The existence of the first condition would be of extreme interest in view of the fact that *Codium* is a diploid alga, and the latter condition would indicate that some species of *Codium* either multiply asexually by diploid zoospores or possess an alternation of generations.

As is well known, many of the species and varieties of *Caulerpa* intergrade to a degree that is perplexing to the systematist. Dostál (1929, 1945) has shown not only that this genus can be grown successfully in culture but that it lends itself to experimental work. It thus appears likely that a cultural study of the species will give a better indication of the degree of flexibility of the various entities than can be obtained from observation of plants from the sea alone.

In the family Codiaceae, which comprises some seventeen genera, reproductive organs have been observed in *Codium* and *Halimeda* only. It seems likely that in the other genera, the reproductive cells are formed in the undifferentiated thallus as they are in *Caulerpa*. Constant observation of these plants in culture may give an answer to this strange phenomenon.

PHAEOPHYTA[7]

Much of our knowledge of the life cycles of brown algae has been acquired in comparatively recent times. In general, this information was not forthcoming until it had been shown that the complete cycle of development could not be learned unless the plants were grown in culture. To Sauvageau, especially, we are indebted for leadership in this approach to problems relating to the life cycles of brown algae. Among botanists in general he is perhaps best remembered for his epoch-making discovery in 1915 of an alternation of heteromorphic generations in members of the order Laminariales. Over a period of some thirty years previous to his death in 1936, he contributed a long series of important papers on the ontogeny of a large number of brown algae.

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[7] See footnote, p. 79.

In 1933 Kylin published a paper which formed an important landmark in the advancement of knowledge of the brown algae. In addition to contributing new information on the life cycles of some of these plants, he utilized the available knowledge of their structure and reproduction to establish a new system of classification.

In earlier systems, the classification of the higher categories of brown algae was largely based on structure. Although structure is still of great importance in the separation of related species, genera, families and in a few instances even orders, the system of Kylin offered a notable departure in that it was to a large extent based upon life cycle. In view of the transcendent value of the type of ontogeny as an indicator of phylogenetic affinities in lower organisms, the system of Kylin must be considered as portraying more accurately the basic relationships among brown algae than do earlier systems.

On the basis of life history, Kylin divided the Phaeophyta into three classes: Isogeneratae, Heterogeneratae and Cyclosporeae. As the names imply, the class Isogeneratae includes forms which show an alternation of isomorphic generations and the Heterogeneratae comprise forms which possess an alternation of heteromorphic generations, with the sporophyte usually macroscopic and the gametophytes microscopic in size. The Cyclosporeae include the single order Fucales, the members of which lack an alternation of generations. The plants are diploid and meiosis occurs at gametogenesis. Kylin placed five orders (Ectocarpales, Sphacelariales, Cutleriales, Tilopteridales and Dictyotales) in the Isogeneratae and six in the Heterogeneratae (Chordariales, Sporochnales, Desmarestiales, Punctariales, Dictyosiphonales and Laminariales). However, Papenfuss (1947) has included the Punctariales in the Dictyosiphonales. A diagrammatic representation of the slightly modified system of Kylin is given in figure 5.

In the brown algae having an alternation of generations, meiosis occurs in the unilocular sporangia which are formed by the diploid asexual generation. The haploid spores that are later formed in these sporangia produce gametophytic plants, which may be isogamous, anisogamous or oögamous (fig. 5). In the Heterogeneratae, the gametophytes are filamentous and microscopic; and in two of the orders, the Chordariales and the Dictyosiphonales, the zygotes usually produce filamentous plantlets which resemble the gametophytes. These diploid filaments directly form new sporophytes as lateral outgrowths or they form plurilocular sporangia whose zooids in turn give rise to filamentous stages; and in certain instances several generations of such plantlets (plethysmothalli) may be produced.

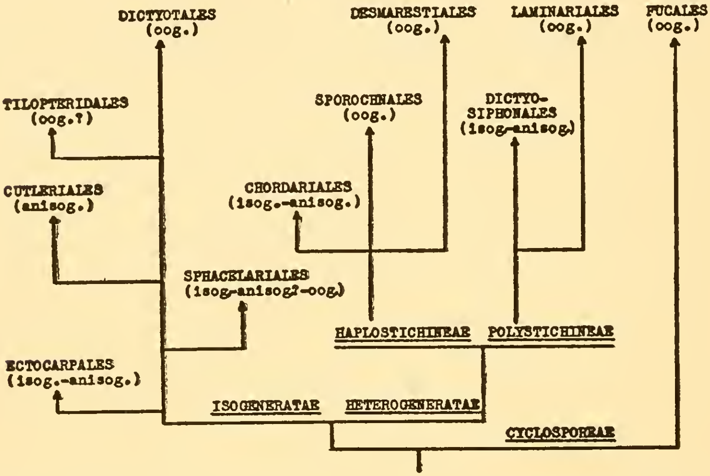

Fig. 5—Diagrammatic representation of the probable interrelationships of the orders of the Phaeophyta.

In many members of the Ectocarpales, Sphacelariales, Chordariales and Dictyosiphonales, the sporophytes also form plurilocular sporangia. No meioses occur in these sporangia. The diploid zoospores that are produced in them serve as accessory reproductive cells of the asexual generation. In the Chordariales and Dictyosiphonales these zooids form plethysmothalli which sooner or later produce sporophytes as lateral outgrowths. In certain members of these two orders the life history thus includes two kinds of filamentous plantlets—gametophytes and plethysmothalli—in addition to the conspicuous sporophytic phase upon which the name of the species is based.

For a long time the method of survival of ephemeral brown algae constituted a perplexing problem, and it was only with the advent of cultural studies that it was learned that these algae persist in a filamentous condition during their period of absence from the flora of a given region.

Although the system of classification of the orders and of many of the families of the Phaeophyta appears to be based on reasonably secure foundations, large gaps remain in our knowledge of the life cycles of these algae.

Many of the genera in the orders Ectocarpales, Chordariales and Dictyosiphonales are in particular need of study. To mention but one example, in the family Myrionemataceae of the order Chordariales only one species, *Myrionema strangulans* Grev., has been investigated (Kylin, 1934). It was found to possess an alternation of heteromorphic generations and on this basis the entire family, which includes a comparatively large number of algae, was placed in the Chordariales. It is not unlikely that some of the forms will be found to possess an alternation of isomorphic generations and hence show themselves as belonging to the Ectocarpales.

In the Sporochnales, the peculiar parthenogenetic (?) development of the egg, in the two species that have been investigated thus far (Sauvageau, 1926, 1927), is a matter which calls for further study.

The preceding account summarizes the main outlines of our present knowledge of the life cycles of brown algae. It has also been pointed out that much of this knowledge was obtained from cultural studies, and it was shown how this information has been utilized in the establishment of a new system of classification. In concluding my remarks on brown algae it may be of interest to refer briefly to two papers by Schreiber that illustrate particularly well the usefulness of cultures in the study of questions which as yet have received very little attention.

Schreiber (1935) succeeded in culturing plants of *Dictyota dichotoma* (Huds.) Lamour. which in stature approached those occurring in nature. Branches that are removed from the plants regenerate complete thalli and thus make it possible to obtain may individuals of identical genetic constitution. Schreiber also succeeded, in a number of instances, in raising to sexual maturity the four plants developing from a tetrad of spores in *Dictyota,* and found that invariably two of them were male and two female individuals, which proved conclusively that sex was determined genotypically.

From this work it is apparent that *Dictyota* offers immense possibilities (as perhaps do related genera) for the study of genetic problems in the brown algae.

Schreiber (1930) has also demonstated experimentally that in *Laminaria saccharina* (L.) Lamour. sex determination is genotypic. Furthermore, he found that the eggs of some species of *Laminaria* will develop parthenogenetically. The resulting sporophytes grew well but were atypical. Whether or not they were capable of producing reproductive organs was not determined. Experiments designed to determine if related species would hybridize gave negative results. No cross fertilizations occurred. Schreiber expresses the opinion that an algal genus such as *Laminaria* probably represents a much more comprehensive entity than a genus such as *Cirsium* in the Compositae and phylogenetically perhaps is the equivalent of the entire family Compositae. Although Schreiber's failure to obtain cross fertilization in *Laminaria* does not justify such a sweeping statement, it does emphasize the need for many more studies of this kind in the algae.

RHODOPHYTA[8]

Although a considerable amount of work has been done on the development in culture of the sporeling stages of red algae (Killian, 1914; Kylin, 1917; Chemin, 1937; Inoh, 1948; and many other investigators), the results obtained in general have not been as significant as those in the green and especially the brown algae. From the following review of a few of the more outstanding contributions it will be apparent, however, that rich rewards may be in store for those who resort to cultural methods in studies dealing with problems in the morphology and taxonomy of red algae.

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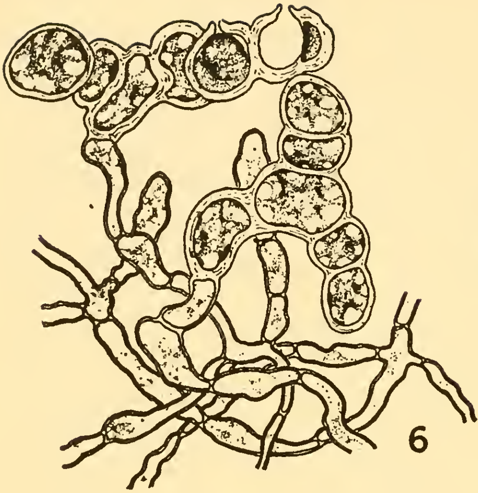
[8] See footnote, p. 79.

In a recent issue of NATURE, Drew (1949) reported upon some very interesting observations on the development, in culture, of the spores of *Porphyra.* She found that the spores gave rise to filamentous stages which, if allowed to grow on mollusc shells, produced growths that in every respect resembled those of the heretofore accepted monotypic genus *Conchocelis* (fig. 6). From this she concludes that this alga of uncertain systematic position represents a phase in the life history of *Porphyra.*

It is not clear from the account of Drew whether she was culturing the monospores or the carpospores of *Porphyra,* but in the light of earlier studies by Reinke (1878), Berthold (1881), Kylin (1921, 1946), Ueda (1929), Rees (1940) and others on *Porphyra* and *Bangia* it seems safe to conclude that she was dealing with carpospores. From the work of these investigators it is known that the monospores give rise directly to new plants of *Porphyra* or *Bangia,* whereas the carpospores produce filamentous stages (not previously associated with *Conchocelis*) which presumably form monospores whereby the *Porphyra-* or *Bangia-*stages are reestablished. Dangeard (1931) has illustrated stages which indicate that in certain instances the filamentous phase of *Porphyra,* however, directly produces *Porphyra* plants as outgrowths from the filaments.

In Drew's cultures the *Porphyra*-stage had not yet been reestablished, but the study is still in progress and it is to be hoped that definite information will be forthcoming on this controversial point. In this conection, it is of interest to note that Jao (1937) has given some very convincing evidence of the formation of monosporangia by *Conchocelis* (fig. 6).

Observations which in some respects are even more remarkable than those of Drew also have been made by J. and G. Feld-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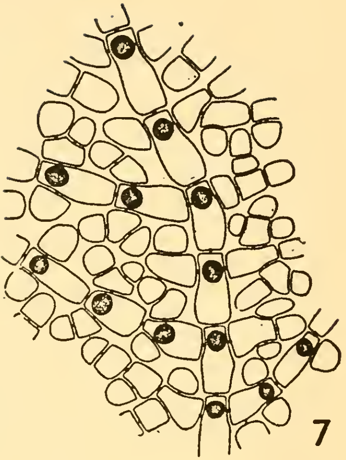
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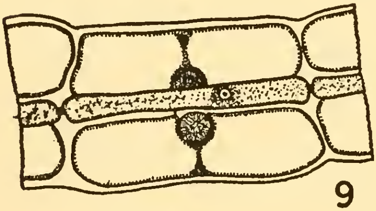
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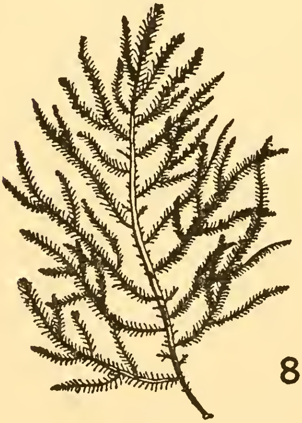
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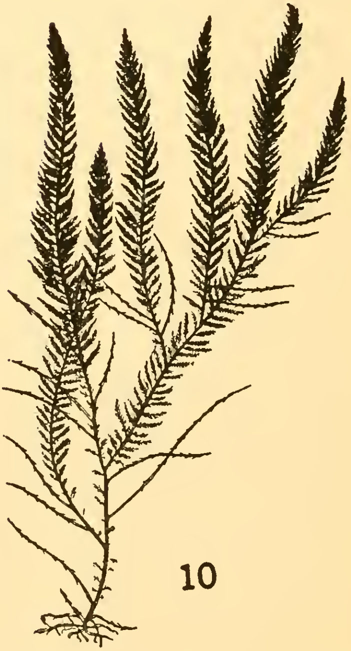
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Figs. 6-10. Fig. 6—*Conchocelis rosea,* vegetative filaments of thallus and monosporangia. (× 550). From Jao, 1937. Fig. 7—*Hymenoclonium serpens,* portion of thallus. (× about 350). From J. and G. Feldmann, 1942. Fig. 8—*Bonnemaisonia asparagoides,* habit of plant. (× about 2/5 natural size). From Newton, A handbook of the British seamann (1942 and earlier papers) with respect to the life cycles of certain members of the order Nemalionales. On the basis of cultures established from carpospores, they claim: (1) that *Hymenoclonium serpens* Batt. (fig. 7), which is the type species of the genus, represents a tetrasporic phase in the life cycle of *Bonnemaisonia asparagoides* (Woodw.) C. Ag. (fig. 8); (2) that *Falkenbergia rufolanosa* (Harv.) Schmitz (fig. 9), which is the type species of the genus, represents a tetrasporic phase in the life cycle of *Asparagopsis armata* Harv. (fig. 10); and (3) that *Trailliella intricata* Batt., which is the type and the only species of the genus probably represents a tetrasporic phase in the life cycle of *Bonnemaisonia hamifera* Hariot. *Hymenoclonium, Falkenbergia* and *Trailliella* have all been genera of uncertain systematic position, and if J. and G. Feldmann are correct, these three genera could be disposed of in a most satisfactory way.

Although the illustrations given by J. and G. Feldmann convincingly suggest that the filaments which develop from the carpospores of *Bonnemaisonia asparagoides* and *Asparagopsis armata* are identical with plants of *Hymenoclonium serpens* and *Falkenbergia rufolanosa,* respectively, the conclusion that these pairs of species represent phases in the life of the same plant can not be regarded as absolutely established until *Bonnemaisonia asparagoides* and *Asparagopsis armata* have been obtained in culture from the tetraspores of free-living plants of *Hymenoclonium serpens* and *Falkenbergia rufolanosa,* respectively. In addition, the cytology of the two kinds of plants calls for study. This is particularly necessary in view of the cytological work of Svedelius (1933) on both *Bonnemaisonia asparagoides* and *Asparagopsis armata.* His observations indicate that in both of these plants meiosis occurs immediately after fertilization. The carpospores as well as the *Hymenoclonium-* and *Falkenbergia*-stages which issue from them should thus be haploid. Consequently, it must be supposed, as do J.

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weeds, 1931. Fig. 9—*Falkenbergia Hillebrandii* (= ? *F. rufolanosa*), longitudinal section of portion of the polysiphonous thallus. (× 430). From Kylin, Anatomie der Rhodophyceen, 1937. Fig. 10—*Asparagopsis armata,* habit of plant. (× about 5/13 natural size). From Harvey, Phycologia Australica, Vol. 4, 1862.

and G. Feldmann, that the tetraspores of these plants are the products of mitosis of haploid nuclei instead of meiosis as they usually are in red algae and elsewhere in the plant kingdom.

It is well known that in a number of red algae tetrasporangia and sexual organs at times occur on one and the same thallus (Svedelius, 1914; Kniep, 1928; and Drew, 1944). This suggests that in certain instances typical tetrasporangia (with the content divided into a tetrad of spores) actually may be formed by haploid plants. To the knowledge of the writer, however, no cytological study has been made to explain the anomalous situations presented by such plants or by the *Bonnemaisonia-Hymenoclonium* and *Asparagopsis-Falkenbergia* alliances. The sporangia occurring on the sexual plants of *Nitophyllum punctatum* (Stackh.) Grev. form a single uninucleate spore (Svedelius, 1914); and it has been shown by Yamanouchi (1906) and by Lewis (1909) that the sporangia which are formed on certain sexual plants of *Polysiphonia flexicaulis* (Harv.) Collins and *Griffithsia globulifera* Harv., respectively, also do not become divided into tetrads, although the behavior of the nucleus in these sporangia could not be determined precisely.

Kylin (1945) repeated the work of J. and G. Feldmann with respect to the development in culture of the carpospores of *Bonnemaisonia asparagoides* (Woodw.) C. Ag., and has confirmed their observations that the carpospores produce filamentous stages which resemble *Hymenoclonium serpens* Batt.; and he was inclined to accept the view that the latter is merely a stage in the life of the former. However, in Kylin's cultures, typical *Bonnemaisonia* plantlets developed as outgrowths from the *Hymenoclonium*-like filaments. The anlages of the *Bonnemaisonia* plantlets somewhat resembled tetrasporangia, which fact caused Kylin to conclude that J. and G. Feldmann had mistaken these structures for tetrasporangia. In a subsequent article J. and G. Feldmann (1946) adhere to their original opinion that these structures are tetrasporangia, the four spores of which in Kylin's cultures, according to their interpretation, germinated *in situ* to produce collectively a *Bonnemaisonia* plant. Evidently the problem is in need of further study.

The use of cultures most likely will assist in finding an explanation of a variety of other important questions relating to the life cycles of red algae. For example, in the family Acrochaetiaceae of the order Nemalionales, certain species form only sexual organs and monosporangia whereas others produce only tetrasporangia. As has been pointed out by Papenfuss (1945), it is not improbable that some of these species represent a generation in the life cycle of others; but this can be determined only from cultural and cytological studies.

Among the larger red algae, many of which are common and have been under observation for a long time, there are likewise species which appear to be represented in nature either by sexual individuals only (several Dumontiaceae, *Schizymenia*, and others), or by asexual individuals only (*Halosaccion, Hildenbrandia,* and others). Promise of an ultimate solution to some of these peculiar phenomena is furnished by the excellent results of Dammann (1930) in her study of *Halarachnion ligulatum* (Woodw.) Kütz. No certain records exist of the occurrence in nature of tetrasporangial plants of this species. Nonetheless they were obtained by Dammann in cultures which were started from carpospores.

In many respects only a beginning has been made in the cultural approach to problems in the red algae. In this review, brief mention has been made of some of the work that has been done and a few of the problems that might be studied by this method of attack. Although consideration has been given only to questions which directly bear upon the life cycles and indirectly on the systematics of red algae, the usefulness of members of this group in experimental studies should be explored also. The majority of red algae include three generations in their life cycle: a haploid gametophyte, a diploid carposporophyte which is parasitic on the gametophyte and a free-living, diploid tetrasporophyte. It would be of great interest to see whether the young carposporophyte would grow in culture and, if it does, whether it is capable of producing a tetrasporophyte directly.

APPENDIX

MEDIA FOR THE CULTURING OF ALGAE[9]

1. Chu No. 10, with modified iron source. (Gerloff, Fitzgerald and Skoog)

Compound	*Grams per Liter*
$Ca(NO_3)_2$	0.040
K_2HPO_4	0.010
$MgSO_4 . 7H_2O$	0.025
Na_2CO_3	0.020
Na_2SiO_3	0.025
Ferric Citrate	0.003
Citric Acid	0.003

In his paper (Jour. Ecol., 30: 284-325. 1942) Chu specifies $FeCl_3$ as the iron source in this solution. We found ferric citrate, however, to be superior to the ferric chloride. In his 1948 paper (Symbol. Bot. Upsal., 10: 1-149) Rodhe reports similar results and, in addition, that the availability of iron is increased if an equal amount of citric acid is added with the ferric citrate. We have followed Rodhe's suggestion on the use of citric acid. A ferric citrate-citric acid stock solution is prepared fresh each week. Although Chu was primarily interested in green algae and diatom culture, we have found his modified No. 10 solution suitable for the continuous culture of twenty-four species of blue-green algae.

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[9] Submitted by authors as indicated.

2. Nutrient Solution for Mineral Nutrition Studies of Blue-Green Algae. (Gerloff, Fitzgerald and Skoog.)

This medium contains the same essential elements as the modified Chu No. 10 solution, but is superior to it for nutrition experiments inasmuch as the concentration of each major essential element may be varied independently of the others.

Compound	*Grams per Liter*
$NaNO_3$	0.0413
Na_2HPO_4	0.0082
KCl	0.0086
$MgCl_2 . 6H_2O$	0.0209
Na_2SO_4	0.0146
$CaCl_2 . 2H_2O$	0.0359
Ferric Citrate	0.003
Citric Acid	0.003
Na_2CO_3	0.0200
Na_2SiO_3	0.0250

Hoagland's A-Z solution is added to the culture solution at one twenty-fifth the strength specified for higher plants.

3. Inorganic Media. (H. C. Bold)

A. Beijerinck's Solution (Zent. Bakt. Par. Infek. II, 4: 785-787. 1898).

Compound	*Grams per Liter*
Distilled Water	1000.0 ml
NH_4NO_3	0.5
K_2HPO_4	0.2
$MgSO_4 . 7H_2O$	0.2
$CaCl_2 . 2H_2O$	0.1

B. Modified Bristol's Solution (Bold, Bull. Torr. Bot. Club, 76: 101-108. 1949).

This solution is prepared as follows: six stock solutions, 400 ml. in volume are employed, each containing one of the following salts in the concentration listed:

$NaNO_3$	10.0 g.
$CaCl_2$	1.0
K_2HPO_4	3.0
KH_2PO_4	7.0
$MgSO_4$	3.0
NaCl	1.0

10 ml. of each stock solution are added to 940.0 ml. of Pyrex-distilled water. To this are added also one drop of a 1.0 per cent $FeCl_3$ solution and 2 ml. of minor elements solution (Trelease and Trelease, Amer. Jour. Bot., 22: 520-542. 1935).

4. Organic Media. (H. C. Bold)

A. Volvox Solution (Moewus, Gruppe. Zeit. f. Induk. Abst. u. Vererb., 78: 418-522. 1940).

Autoclave 1 Kg. garden soil in 1 liter of distilled water at 15 lbs. pressure for 1 hour. Cool, decant and filter off liquid extract "E." To make definitive solution:

Distilled Water	300.0 ml.
5% KNO_3	1.0 ml.
"E"	30.0 ml.

B. "Bristol's-Yeast" Solution (Bold).

Add 1 gram per liter of Difco Yeast Extract per liter of modified Bristol's solution and sterilize.

C. "Bristol's-Liver" Solution (H. C. Bold).

Make a 10 ml. vial of Armour's Crude Liver Liquid Parenteral up to 100 ml. Add 5 ml. of this to each liter of modified Bristol's Solution and sterilize.

Comments: No. 3-A, 3-B and 4-A (above) are extremely useful in uni-algal culture. No. 4-B and 4-C (above) are most useful in effecting more rapid growth of bacteria-free cultures. The possibility should be considered that cultures grown for long periods in organic media may mutate or become adapted. Any of the solutions listed above may be solidified by the addition of 1.0-1.5% agar.

5. Culture Solution V. (Rodhe, Symbol. Bot. Upsal., 10 (1): 1-149. 1948).

	mg per L		*mg per L*	*mg per ml in stock solution*	*ml of stock solution per liter of medium*
Ca	4.0	$Ca(HCO_3)_2$	16.0	solid	10 mg $CaCO_3$*
Mg	5.0	$MgSO_4$	25.0	1	25
Na	7.5	Na_2SiO_3	20.0	1	20
K	2.2	K_2HPO_4	5.0	1	5
Cl	13.3	NH_4Cl	20.0	1	20
SO_4	20.0	$MgSO_4$	25.0		
HCO_3	12.0	$Ca(HCO_3)_2$	16.0		
N	5.25	NH_4Cl	20.0		
P	0.89	K_2HPO_4	5.0		
Fe	0.18	Ferric Citrate—Citric Acid	1.0 & 1.0	0.1 & 0.1	
Mn	0.01	$MnSO_4$	0.03	0.1	10†
Si	4.6	Na_2SiO_3	20.0		0.3

* "The dissolution of $CaCO_3$ can be accelerated by bubbling through CO_2."

† "The iron stock solution is autoclaved separately and is added to the sterile culture solution. It must be kept in the dark."

6. Culture Solution VIII for Plankton Algae. (Rodhe, l.c.)

	mg per L		*mg per L*	*mg per ml in stock solution*	*ml of stock solution per liter of medium*
Ca	14.7	$Ca(NO_3)_2$	60.0	10	6.0
Mg	1.0	$MgSO_4$	5.0	1	5.0
Na	7.5	Na_2SiO_3	20.0	1	20.0
K	2.2	K_2HPO_4	5.0	1	5.0 *
SO_4	4.0	$MgSO_4$	5.0		
N	10.2	$Ca(NO_3)_2$	60.0		
P	0.89	K_2HPO_4	5.0		
Fe	0.18	Ferric Citrate—Citric Acid	1.0 & 1.0	0.1 & 0.1	10.0 †
Mn	0.01	$MnSO_4$	0.03	0.1	0.3
Si	4.6	Na_2SiO_3	20.0		

* "The phosphate stock solution is autoclaved separately and added to the culture solution after its autoclaving; otherwise precipitation (calcium phosphate ?) can accidentially appear after autoclaving."

† "The iron stock solution is likewise autoclaved separately and added to the culture solution after its autoclaving; otherwise the ferric citrate is hydrolysed, the iron being precipitated as $Fe(OH)_3$."

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[10] This paper contains an account of the development of synthetic media for a fresh-water and a marine *Chlamydomonas,* and a general description of the assay of vitamin B_{12} by means of *Euglena gracilis.*

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